trends in BIOTECHNOLOGY

ISSN 0167 9430

REFERENCE EDITION 1997

including author and subject index

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Published by:
Elsevier Trends Journals,
68 Hills Road,
Cambridge,
UK CB2 1LA

Printed in the United Kingdom

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REFERENCE EDITION
Volume 15
1997

ELSEVIER
TRENDS
JOURNALS

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TRENDS IN BIOTECHNOLOGY – REFERENCE EDITION

Published by Elsevier Trends Journals

The reference edition of **Trends in Biotechnology** is a compilation of review and other articles reprinted from the regular monthly magazine edition. For citation purposes, readers are asked to note that the page numbers of reprinted articles are common to both editions.

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A drug screening program for ion channels expressed in yeast

Workshop

The function of some ion channels, when expressed in yeast, is dependent on a proton gradient established by the yeast plasma membrane H^+-ATPase. The activity of the heterologous ion channel can be assessed by monitoring growth properties of the strain and by microphysiometry, which measures changes in the rates of proton efflux (extracellular acidification). These two assays function in concert as a rapid system for screening and evaluating compounds that modulate channel function.

It is the combination of advances in electrophysiological techniques and molecular cloning that has provided the opportunity and rationale for drug screening programs that focus on distinct ion-channel targets. For example, the coupling of heterologous expression of a specific channel-encoding gene in *Xenopus laevis* oocytes with voltage analysis of currents derived from the expressed channel has enhanced the process of identifying compounds that modulate that channel[1]. Given the complex array of currents comprising the cardiac action potential, this approach has many prospects. While voltage clamping provides the most sensitive method for evaluating the effects of test compounds on channel function, the number of compounds that can be rapidly evaluated by this technique is low. In some cases, expression of the target channel in a microorganism can expand the screening capacity and serve as a primary drug screen to maximize compound evaluation in concert with electrophysiological methods. We describe an application of microphysiometry as an assay with moderate capacity, yet high specificity, to augment primary assays based on cell growth.

The basis for using microphysiometry on yeast cells is the proton-exporting ATPase (H^+-ATPase), a component of the fungal plasma membrane that is involved in the regulation of intracellular pH. The proton-extruding action of the H^+-ATPase generates an electrochemical proton gradient that preserves ion balances and facilitates the uptake of other ions and nutrients through secondary transport systems[2]. The proton gradient is essential for cellular function as its perturbation results in cell death[3–5].

The microphysiometer provides a uniform system for assessing channel functions and specific drug interactions that is suitable for drug screening (see Box 1). It contains a flow chamber in which cells are immobilized and a silicon sensor that measures pH changes in the medium bathing the cells[6,7]. Changes in pH, expressed as rates of extracellular acidification, reflect alterations in metabolic activity and transmembrane proton flux associated with intracellular pH homeostasis[8]. Alterations to the yeast proton gradient can be measured by recording extracellular acidification rates through microphysiometry. These measurements provide a sensitive means to analyze the function of certain ion channels expressed in yeast.

The capabilities of genetic manipulation in the yeast *Saccharomyces cerevisiae* have allowed the isolation of genes encoding functional homologs from other organisms that complement yeast mutants. This approach was used to identify cDNAs encoding K^+ channels from the plant *Arabidopsis thaliana* that restored K^+ uptake in a yeast strain defective in K^+ transport[9,10]. This work opened up the possibility of expressing ion channels from other organisms and offered the potential of adapting genetically stable strains into high-capacity screens for identifying modulators of ion uptake.

Using a similar yeast mutant, complemented for K^+ uptake with the cardiac inwardly rectifying K^+ channel 1 (IRK1)[11], we have illustrated a process for evaluating compounds that modulate channel functions beginning with effects on growth. The strain grows on standard medium because of the expressed K^+ channel (Fig. 1a). Its growth is inhibited in the presence of cesium, a known blocker of IRK1 channel function[12], whereas the growth of control strains is unaffected by cesium (Fig. 1b). Selective growth inhibition of the strain expressing the ion channel serves as a primary assay for compounds that might modulate channel function. Lead compounds identified in this primary assay can then be further evaluated using microphysiometry: a rapid decrease in acidification rates upon cesium exposure is indicative of a reduction in proton extrusion (Fig. 2). Cesium had no effect on proton flux in control strains, indicating its selectivity for the ion channel. Upon removal of the cesium block, there occurred a rapid, transient increase in acidification rates in chambers containing the channel expressing cells, indicating that reversal of the channel block was accompanied by restoration of proton export. The magnitude of the response to cesium exposure is

Box 1. Microphysiometer analysis

Extracellular acidification rates were measured using a microphysiometer[6–8]. This instrument contains microvolume flow chambers in contact with a pH-sensitive silicon sensor. Cells are immobilized in an agarose plug that is then inserted between two porous membranes. The lower membrane is in direct contact with the sensor while the upper membrane is in contact with the flow of fresh medium. By periodically interrupting the flow of medium through the chambers, the sensors record alterations in pH which reflect changes in rates of extracellular acidification. These rates are sensitive to changes in metabolic activity (such as excretion of acidic metabolites) and to the transmembrane proton flux associated with intracellular pH homeostasis. The instrument has previously been used with mammalian cells to monitor receptor activation and signal transduction[7].

The extracellular acidification rates of yeast strains described here were measured by immobilizing approximately 4×10^5 log-phase yeast cells in an agarose plug. Flow chambers were maintained at 30°C and perfused at a rate of 100 μl min^{-1} with the appropriate minimal medium. Acidification rates were measured at 80 s by interrupting the flow of media for 10 s and are expressed as percentage values of basal rates before exposure to various ion-channel modulators. Although basal rates can vary between experiments, the percentage change in rates observed in response to test compounds is reproducible.

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PII: S0167-7799(96)10073-1

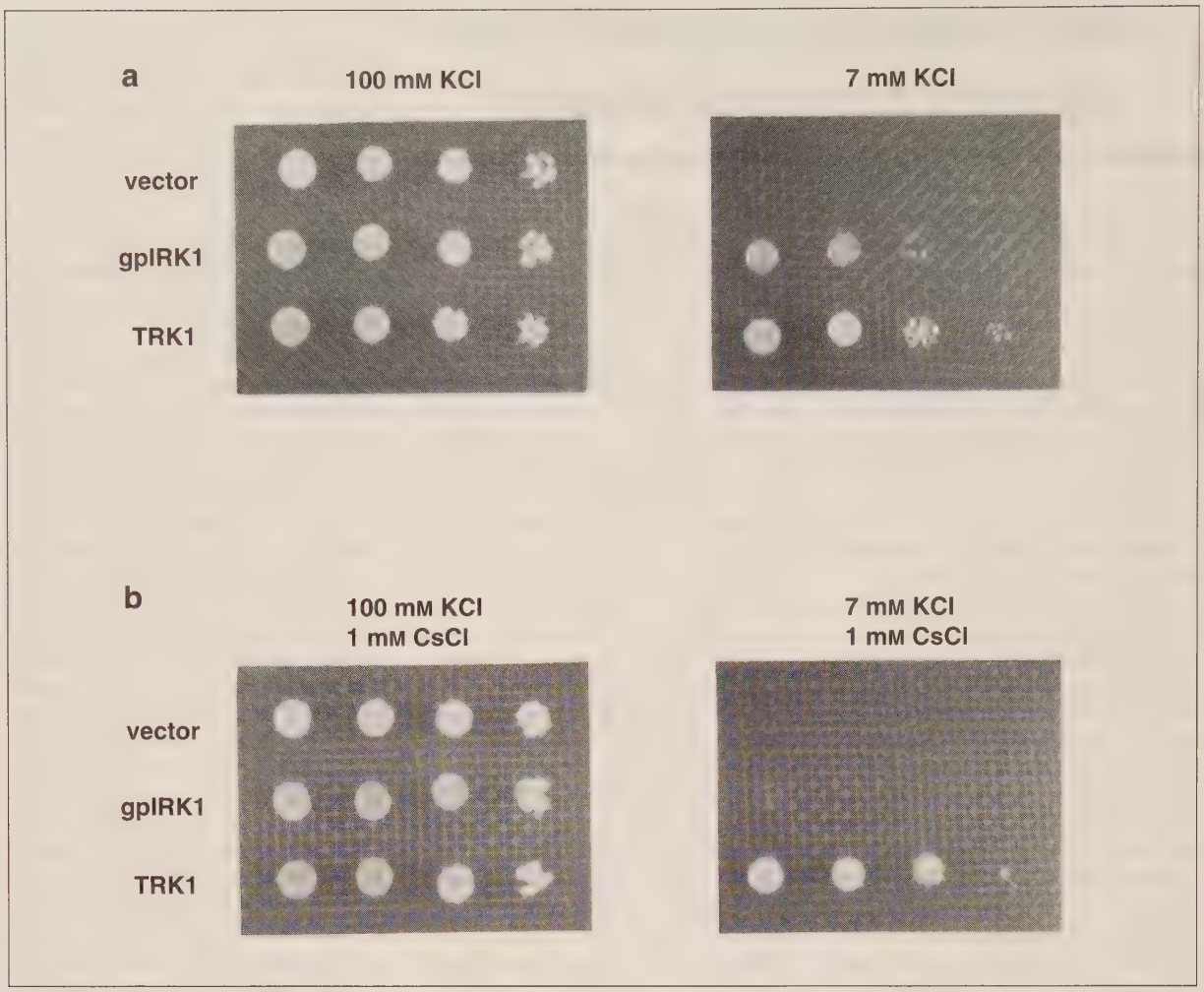

Figure 1

(**a**) A yeast strain expressing an inwardly rectifying K^+ channel, gpIRK1, grows on standard yeast medium (7 mM KCl), whereas a control strain containing the empty vector (designated vector) requires medium supplemented with 100 mM KCl for growth. As expected, the control strain expressing the yeast K^+ transporter, TRK1, grows on standard medium. Details are described in Ref. 11. Reproduced, with permission, from Ref. 11. (**b**) Growth of the strain expressing the gpIRK1 ion channel is selectively inhibited in the presence of CsCl, whereas growth of a strain expressing the yeast K^+ transporter TRK1 is unaffected.

directly affected by the external K^+ concentration, which is expected if K^+ and cesium compete for the same binding site in the pore region of the channel. The decrease in transmembrane proton flux observed upon blocking K^+ uptake with cesium probably reflects the stoichiometric relationship (1:1) between K^+ uptake and H^+ export in *S. cerevisiae*[13]. As measured by voltage clamping, inhibition of current produced from this channel with cesium provides the definitive test of ion-channel blockade and serves as a final assay in confirming the ion-channel-modulating properties of test compounds (Fig. 3).

We used the microphysiometer in a screening program to identify inhibitors of M2 protein, the proton-conducting ion channel from influenza A virus. Expression of high levels of the M2 ion channel in a wild-type yeast strain impairs growth, apparently by altering the electrochemical proton gradient established by the H^+-ATPase. Growth inhibition was used to design a primary screen to detect inhibitors of M2 function and uncovered a novel inhibitor, BL-1743, from a chemical library of 10^5 compounds[14]. The antiviral activity of BL-1743 was confirmed in a whole virus assay. Analysis of acidification rates using microphysiometry in the presence of BL-1743 indicated that channel blockade results in an increase in acidification rates consistent with the hypothesis that M2 perturbs the proton gradient. The microphysiometer also demonstrated the reversible inhibitory nature of BL-1743 (Ref. 15). Similar data on BL-1743 inhibition were obtained by voltage analysis of oocytes expressing the M2 channel[16].

The microphysiometer has also been used to measure the activity of the Na^+/H^+ proton antiporter encoded by the *sod2* gene of *Schizosaccharomyces pombe*[17], which opens up the possibility of expressing genes encoding vertebrate Na^+ exchangers or ATPases to complement relevant yeast mutants. Other proteins, such as the *vpr* gene product of HIV-1, may be attractive candidates for a screening program similar to that designed for M2; expression of *vpr* in yeast causes growth arrest and osmotic sensitivity[18] and an ion-channel function has been observed[19]. Assessment of ion-channel activity using the system described here requires a relationship between the expressed channel and proton flux. In this regard, changes

in extracellular acidification measured by the microphysiometer provide an indirect measurement of the action of the heterologous ion channel in yeast.

In comparison with other available methods for measuring ion flux in yeast, such as ion-selective electrodes or patch clamping[20], the flow chamber configuration of the microphysiometer permits the rapid introduction and removal of test compounds, and maintains a constant extracellular environment through continuous supply of fresh medium to the cells. In contrast to patch clamp techniques, which provide a direct assessment of flux through an ion channel, the microphysiometer records alterations in transmembrane proton flux resulting from modulation of an ion channel expressed in yeast. Nevertheless, results obtained with the microphysiometer provide analogous data to those obtained by patch clamping. This application provides a uniform system for assessing channel functions and specific drug interactions. As a component of a screening program centered on heterologous ion channels in yeast, the microphysiometer offers a process with moderate capacity for screening compounds that modulate channel function. It has the potential to classify genuine lead compounds identified from primary growth-based screens. The assay is rapid, dose–response relationships are readily determined and up to eight compounds can be analyzed simultaneously with a multichannel instrument. Lead compounds identified using the growth assay in combination with the microphysiometer serve to direct the focus of subsequent evaluation using more-rigorous electrophysiological methods.

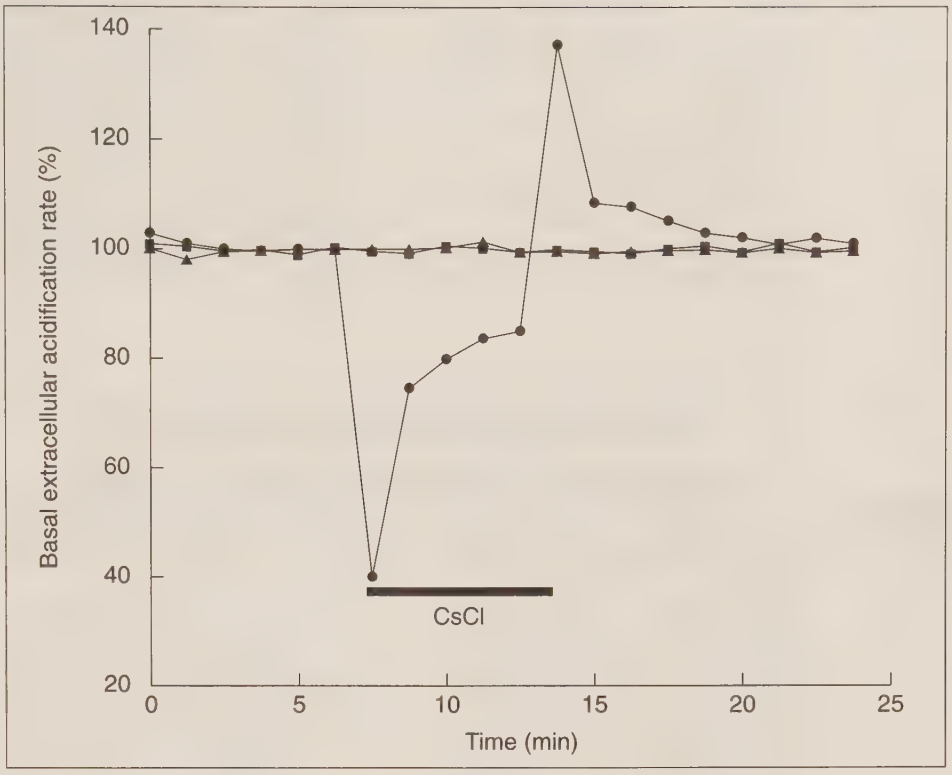

Figure 2

Extracellular acidification rates of yeast cells containing the empty vector (▲), cells expressing TRK1 (■), and cells expressing gpIRK1 (●) were measured using a Cytosensor microphysiometer as described in Ref. 15. Exposure of the cells expressing gpIRK1 to 50 μM CsCl (indicated by the bar) results in a rapid decrease in proton export that is readily reversible upon removal of the channel blocker.

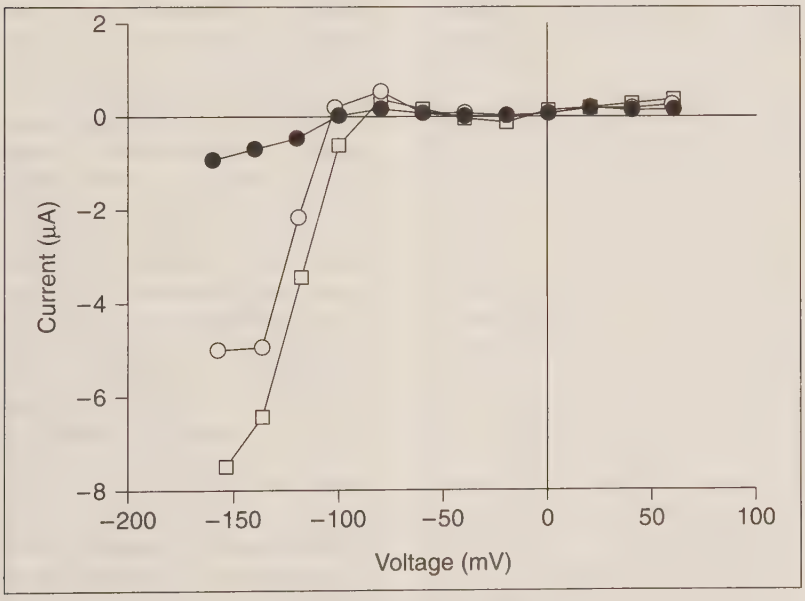

Figure 3

Current–voltage relationship recorded from two-electrode voltage analysis on oocytes expressing gpIRK1 (□). Plots indicate inhibition of gpIRK1 current in the presence of cesium at 10 μM (○) or 100 μM (●). Reproduced, with permission, from Ref. 11.

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Workshop

Avoiding protease-mediated resistance in herbivorous pests

In the course of their evolution, plants have developed a multitude of defense mechanisms against herbivorous pests and pathogens, including various kinds of defense-related proteins[1]. Owing to recent developments in plant biotechnology, DNA sequences encoding novel, foreign proteins with protective properties can now be integrated into the genome of plants to complement the effect of these naturally occurring proteins. Genetically modified plants expressing δ-endotoxins from the soil bacterium *Bacillus thuringiensis* (Bt toxins), for instance, show a high resistance status against herbivorous insects[2,3], making this antibiotic-based control strategy already suitable for commercialization. The genes of plant defense proteins, such as protease inhibitors (PIs)[4–7], α-amylase inhibitors[8], lectins[9] and chitinases[10], have also been proposed for use in designing plants resistant to pests. Together with Bt toxins, these proteins represent interesting candidate molecules for improving the ability of plants to remain healthy and productive in commercial agricultural systems.

Pests adapt to defense proteins

Despite these promising developments, a gradual decline in the efficiency of transgenic plants can be expected. Effective defense proteins, such as Bt toxins or enzyme inhibitors are single chemical compounds, as are the commonly used synthetic pesticides. The future large-scale commercial use of transgenic plants will exert strong selection pressure on the target pests and probably favor the build-up of resistant populations. To counteract, or at least minimize, the development of resistance in pest populations and to ensure effectiveness of transgenic plants over a long period, such plants must be seen as one component of a more elaborate, multicomponent control system[11].

Understanding the molecular mechanisms by which pests develop resistance and overcome the effect of defense proteins also appears important. A common mechanism for eluding the effects of pesticides involves a change in the affinity between the defense molecule and its target receptor in the pest. Resistance to Bt toxins, for instance, was proposed to be associated with a decreased ability of the bacterial toxin to bind efficiently to its membrane receptor in the insect midgut[12]. In contrast to this kind of resistance, in which the target receptor is no longer recognized, deactivation of the defense protein itself might represent an effective way for the pest to avoid adverse effects. Like any protein, defense proteins are polypeptides potentially recognized as substrates by pest digestive proteases. Altering the structure of defense proteins by proteolysis might allow the pest to be resistant without altering the nature of its target molecules. Recent data suggest that such protease-mediated resistance occurs in herbivorous pests.

Protease-mediated resistance to defense proteins

Resistance to Bt toxins

Forcada and co-workers found that distinct digestive protease complements occurred in two strains of the tobacco budworm: one susceptible and one resistant to the Bt toxin CryIA(b) (Ref. 13). The toxin-resistant strain possessed proteases with increased ability to degrade the mature, active toxin, and a decreased ability to process the nonmature, inactive protoxin. Similar variations of digestive protease complements in Bt-toxin-resistant insects were reported for other species[14,15]. Cleavage-mediated activation of the Bt protoxin CryIA(c) by midgut proteases was visibly affected in a resistant strain of the Indianmeal moth[14], while late instars of the cotton leafworm, resistant to the toxin CryIC, possess digestive proteases that can completely hydrolyze this toxin[15]. Together, these observations suggest that insects can specifically adapt their digestive proteolytic system to the presence of peptidic toxins in the meal.

An alternative scheme for protease-mediated deactivation of Bt toxins was recently reported by Milne and co-workers[16] for the spruce budworm. A protein complex present in the insect midgut and exhibiting protease activity was shown to inactivate the toxin CryIA(a) by precipitation, after forming an insoluble complex with the bacterial protein. This deactivation process was followed by limited cleavage of the toxin, and its subsequent loss of larval toxicity. Interestingly, the midgut soluble content of insects from several different families caused precipitation of the toxin, whereas the midgut content of the silkworm, an insect highly susceptible to the action of CryIA(a), failed to precipitate the toxin[16]. The specificity of Bt toxins would thus not only be determined by their interaction with membrane receptors, but also by their interaction with protease-containing protein complexes acting as a 'molecular trap' in the midgut of target insects.

Resistance to PIs

Proteolysis by pest proteases was also proposed to explain the inefficacy

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PII: S0167-7799(96)10072-X

of certain PIs to alter development of target pests[17]. The inhibitory range of PIs is generally limited to proteases in one of several mechanistic classes, leaving insensitive proteases from other classes free in the surrounding medium after inhibition. Probably owing to coevolution processes, the inhibitory spectrum of PIs in plant–pest systems is apparently even more limited, being restricted to the subclass level[18–21]. As insects usually possess digestive proteases of various classes and subclasses[22], degradation of some plant PIs in the pest midgut appears plausible. Inclusion of different PIs of the cystatin superfamily in the diet of the southern corn rootworm, for instance, resulted in quite varying effects on growth of the insect, although all PIs caused significant inactivation of this insect's proteases *in vitro*[17]. Interestingly, simultaneous inclusion of a second, complementary inhibitor from potato restored activity of the less effective cystatins in the insect gut and their ability to alter growth of the pest. This differential susceptibility of PIs to degradation by nontarget proteases was recently reported for two other coleopteran species: the black vine weevil and the Colorado potato beetle. Nontarget proteases of the black vine weevil, in particular, were shown to differentially affect the structure of two PIs from rice: oryzacystatin I (OCI) and oryzacystatin II (OCII)[23]. Whereas OCI was insensitive to the action of the insect nontarget proteases, OCII was subjected to limited proteolysis at about 1.5 kDa of its C-terminal end, leading to the rapid accumulation of a 10.5 kDa truncated form, which was active but subsequently hydrolyzed by the insect proteases. Similarly, human stefin A, an animal homolog of OCs, was highly susceptible to the action of digestive nontarget proteases of the black vine weevil and Colorado potato beetle[24]. In contrast to OCII, no detectable intermediate was found during hydrolysis of the human inhibitor, suggesting the presence of multiple cleavage sites at the surface of this cystatin.

Resistance to α-amylase inhibitors

Like Bt toxins and PIs, α-amylase inhibitors can be recognized as substrates by digestive proteases of herbivorous pests. For example, midgut proteases of the Mexican bean weevil act on the β subunit of the common bean amylase inhibitor αAI-1 by limited proteolysis[25]. The cleavage was shown to occur specifically at the active site of the polypeptide, rapidly and irreversibly hindering the anti-amylase function of the inhibitor. Although this proteolytic event was not the key factor explaining the insect's resistance to the α-amylase-inhibitor-rich diet, this observation provided additional evidence for the involvement of protease-mediated resistance to defense proteins in herbivorous pests.

A new role for recombinant PIs?

Taken together, these observations clearly show that pests can avoid adverse effects of defense proteins by causing their cleavage. Unwanted proteolysis is a hurdle that must be overcome to ensure the long-term use and benefit of transgenic plants devoted to pest control. Tailoring proteolysis-resistant proteins by genetic engineering might represent one solution to the problem. The common bean α-amylase inhibitor αAI-1, cleaved by midgut proteases of the Mexican bean weevil, is no longer cleaved when the amino acid motif WSY, present at the active site of its β subunit, is changed to the tripeptidic sequence GNV (Ref. 25). However, the rational engineering of defense proteins could be of limited interest in the next few years due to the lack of information about cleavage sites involved in the interaction of pest proteases with their target protein substrates. Alternatively, blocking the activity of pest proteases with large-spectrum PIs represents an interesting avenue. Information about digestive proteases of herbivorous pests is increasing rapidly[22], and several recent studies provided useful data about biochemical interactions taking place between pest extracellular proteases and plant PIs (Refs 18–21,23,24,26).

As a general rule, the inhibitor used should be stable, possess a broad inhibitory spectrum against the pest proteases, and bind tightly to its target proteases. As discussed above, the ability of PIs to remain stable in the presence of nontarget proteases is quite variable[17,23,24], suggesting the importance of choosing inhibitors according to the specific set of proteases found in the pest to be controlled. By minimizing the number of nontarget proteases in the pest midgut, the use of a large-spectrum inhibitor might help ensure its stability. Extending the range of sensitive proteases might also prevent physiological compensation processes by minimizing the appearance of nontarget proteases after feeding[27]. Finally, a high degree of affinity between the inhibitor and its target proteases might help improve the inhibitory effect *in vivo*. By engineering a modified form of OCI with increased affinity for its target proteases and with an improved efficacy in controlling the plant parasitic nematode *Globodera pallida*, Urwin and co-workers[7] demonstrated the importance of using tight-binding inhibitors for an effective control *in planta*. When considering the stability and inhibitory spectrum, affinity must be assessed for each particular pest to control. For instance, OCI shows strong binding activity against extracellular proteases of the root-knot nematode, *Meloidogyne hapla*, whereas its affinity for the proteases of two other species of the same genus, *M. incognita* and *M. javanica*, is significantly lower[28]. Conversely, OCII shows high affinity for proteases of the two latter, closely related species, and a relatively low affinity for the proteases of *M. hapla*.

Future prospects

Provided that tight-binding, large-spectrum inhibitors are found, their integrated use with other defense proteins could result in an efficient two-way control of herbivorous pests. PIs constitute an important class of defense proteins in plants[29] and their potential in plant biotechnology for the control of insect and nematode pests is well established[4–7]. In addition to their potential as control agents, these inhibitors might also be useful agents for protecting recombinant defense proteins of commercial interest, such as Bt toxins, lectins and enzyme inhibitors. They could also prove functional in protecting defense proteins found naturally in host plant tissues, thereby improving efficiency of the plant defense apparatus. A similar protective role for large-spectrum PIs could even be useful in non-plant systems. Recombinant inhibitors could be used, for example, to control the bacterium *Porphyromonas gingivalis* – a causal agent of periodontal diseases – and to help protect defense proteins naturally present in human host tissues. The involvement of extracellular proteases during infection by *P. gingivalis* has been well demonstrated, and PIs naturally present in the host tissues were recently identified as functional substrates for the proteases

of this bacterium[30]. This kind of dual effect of PIs could prove effective in various host–pest and host–pathogen systems.

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Meeting report

Making cells work – metabolic engineering for everyone

Recombinant DNA technology has evolved to the point where it is now feasible for most laboratories to apply it. Apart from its uses in genetics research, this technology has opened up the possibility of engineering metabolism for biotechnological ends, typically in metabolite overproduction[1]. Not only can one overexpress genes of a particular organism, one can also introduce foreign genes to redesign metabolism. Recently, the Engineering Foundation organized a conference on this increasingly popular subject*. Participants were well balanced between academia and industry and came from many countries (roughly half from the organizing country, USA). The topics covered were diverse, ranging from applications of metabolic engineering (ME) to microbial, plant and animal metabolism, to the manufacturing of chiral compounds, with two sessions on tools (experimental and theoretical, and computational). Two workshops took place to debate optimal host cell systems and to define the field of ME. Two poster sessions complemented the subjects of the lectures.

In the keynote lecture, Gerald Fink (Whitehead Institute, Cambridge, MA, USA) sketched what he believes will be the biology of the post-genomics era, which we are entering with the completion of the sequences of the genomes of many organisms. Using examples from the fungal world, he stated the importance of identifying the different behaviour modes, or algorithms, with which organisms perform their functions. We can learn a lot both from the similarities of the algorithms of related organisms and from their differences, but for this we need to identify and understand the expression of the large number of open reading frames whose function is as yet unknown. (Boris Magasanik brilliantly exemplified how complex this will be by describing the regulation of nitrogen-limited growth of *Escherichia coli* and *Saccharomyces* in a memorable after-dinner lecture.) For example, all known fungal pathogens have to switch to a filamentous form before invading the host, and even our old friend *Saccharomyces* is now known to switch to a filamentous mode at some stages of its life cycle. The conditions that influence this switch of 'growth algorithms' could be of major importance for the brewing industry in the control of flocculation of their cultures, and also for the pharmaceuticals industry in the design of drugs to prevent infection by fungal parasites. In the post-genome era the challenge is to identify the function of all sequenced genes and, more importantly, to understand their concerted expression (the phenotype). Other speakers showed specific examples of algorithms that have been decoded and that are now being manipulated to obtain new products (some argued that the word engineering is more relevant to the design of new functions than the optimization of existing ones). The production of antibiotics with aromatic rings by *Streptomyces* can be described by four basic processes (some of which are iterated) (Richard Hutchinson; University of Wisconsin, WI, USA): (1) choose (between substrates); (2) count; (3)

*The meeting 'Recombinant DNA Biotechnology: Focus on Metabolic Engineering' was organized by the Engineering Foundation, and was held in Danvers, MA, USA, 6–11 October 1996.

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PII: S0167-7799(96)30030-9

fold; and (4) cyclize. Novel products have been produced by the combination of various polyketide synthases from different organisms, or even simply by changing the order of their genes in the genome. The understanding of the glycosylation processes in eukaryotes has advanced greatly with the help of techniques such as mass spectrometry and NMR and can also be described by 'algorithms' that can be manipulated by the expression of the appropriate genes (e.g. sialyltransferases), and their physiological manipulation (Nigel Jenkins; De Montfort University, Leicester, UK).

As one would expect in such a conference, many engineered 'high producer' systems were described. The applications ranged widely, from the production of ethanol from office waste paper by engineered *E. coli* with genes from *Klebsiella oxytoca* (Lonnie Ingram; University of Florida, FL, USA) to the production of semisynthetic opiates, also by *E. coli* (Neil Bruce; University of Cambridge, Cambridge, UK). A workshop was organized by Scott Power (Genencor International, Palo Alto, CA, USA) to discuss the selection of an optimal host cell for ME. This was centred on the possibility of constructing a basic minimal host cell that would then be a choice platform for adding metabolic modules (a kind of biological Lego® system) to become a high producer for the product of interest. Even though nobody disagreed with the advantages of such a host (simple, well understood), its construction still seems to be far-off, mainly because our lack of knowledge of the function and, especially, the interaction of most gene products (which tend to interact synergistically), means we cannot decide what to delete from the genome. Even leaving patenting issues aside, it seems that a diverse range of hosts will continue to be used for the foreseeable future.

At first sight, and following what is described in most biochemistry textbooks, one might imagine that to increase a metabolic flux we need only identify the rate-limiting enzyme of the pathway and over express it. However, with the development of metabolic control analysis (MCA) it has become evident that the control of flux is distributed throughout a metabolic pathway (David Fell; Oxford Brookes University, Oxford, UK). Peter Jensen (Technical University, Copenhagen, Denmark) described how MCA can deal with a hierarchical process like gene expression and presented a novel experimental tool that has great potential for allowing the measurement of MCA coefficients. This technique is based on the variable induction of artificial promoters. Promoters of different activities (covering a very wide range) can be selected from a library and placed upstream of the gene of interest, thus fine-tuning its expression (as is needed for the accurate determination of control coefficients). This has the additional advantage that once the optimal promoter activity has been determined, the strain is, in principle, ready for use in the industrial process. MCA was able to help in the selection of targets for overexpression for improving starch accumulation in potato tubers (Michael Burrel; Advanced Technologies, Cambridge, UK). A key point in this work was that no significant effects were observed when only one enzyme (the one with initially the highest control coefficient) was overexpressed. When the concentrations of two enzymes were manipulated, the effect was then satisfactory. This theme recurred throughout the conference. Simulations of the aromatic amino acid production pathway point to the same conclusion, and the best targets for the manipulation are those enzymes that have a high flux-control coefficient and also a low concentration-control coefficient so as to affect the concentration of intermediary metabolites minimally (Greg Stephanopoulos; MIT, Cambridge, MA, USA). Many posters also presented results that revealed large effects on flux only when more than one enzyme was overexpressed.

The message from MCA has been received with open arms by this field, though we are still waiting for the recognition given by textbooks. Other theoretical frameworks are also useful for ME, especially the so-called 'flux analysis', which is mainly a stoichiometric analysis, revealing structural properties of the pathway. This representation can be subject to optimization methods (Vassily Hatzimanikatis; ETH, Zurich, Switzerland), which allows the screening of large sets of possible behaviours. 'Flux analysis' has the advantage that one needs to know only the stoichiometry of the pathway of interest, though even that might not be trivial to determine. ^{13}C NMR determination of fractional isotope enrichment of metabolites can resolve the net- and exchange-fluxes of *Corynebacterium glutamicum* towards L-lysine production (Lothar Eggeling; Forschungszentrum Jülich, Jülich, Germany). Peter Vanrolleghem (University of Gent, Gent, Belgium) described a structured approach to estimate the unknown stoichiometric coefficients. The temporal aspects of steady-state metabolism (mainly the relaxation times after perturbations) should also be taken into consideration for rational improvements (Bernhard Palsson; University of California, San Diego, CA, USA). The combination of theoretical analysis frameworks with experimental determinations of fluxes and metabolite concentrations is the only way to provide a rational means for ME (Jens Nielsen; Technical University, Denmark). With regards to the experimental measurements, many advances will be achieved as novel multivariate, non-invasive methods of analysis become more widely available. On the theoretical side, user-friendly computational tools that perform the required calculations (especially in these days of cheap computational power) are becoming available (Pedro Mendes; University of Wales, Aberystwyth, UK), and will open many new doors to our understanding of metabolism and, therefore, to the engineering of new pathways.

The industrial application of ME is, unfortunately, determined not only by the availability of high-yielding producer strains. Often the overall costs of biological fermentations are higher than those of chemical synthesis, not least for reasons connected with purification costs (Paul Reider; Merck Research Laboratories, Rahway, NJ, USA). The challenge is to devise methods that produce high chemical and enantiomeric purities that are practical on a large-scale, safe, environmentally sound (and this is often where biotechnology is at an advantage over chemical synthesis), efficient and economical. The future of ME lies ultimately in its answers to these problems, rather than just on the characteristics of the actual engineered strains.

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Letter

Naming plant genes

Owing to the burgeoning of idiosyncratic and conflicting names for plant genes, the International Society of Plant Molecular Biology established the Commission on Plant Gene Nomenclature (CPGN) in 1991, with the aim of organizing a common nomenclature for sequenced plant genes. The CPGN operates two offices: one at Rutgers University (Piscataway, NJ, USA), supported by the National Science Foundation and United States Department of Agriculture, and one at the John Innes Centre (Norwich, UK), funded by the European Commission.

The CPGN's goal of a common nomenclature for genes isolated from plants and plant viruses was expanded recently to include genes involved in organelle biogenesis in all organisms except the metazoa. To aid and inform the scientific community of our efforts, we currently provide access on the World Wide Web to Mendel, the CPGN's database <http://probe.nalusda.gov:8300>, and have published *A Guide To Naming Sequenced Plant Genes*[1].

The CPGN's operating premise is straightforward: all genes that encode the same product will be members of the same gene family, and will, therefore, be assigned the same gene name. While the topic of nomenclature is often greeted with indifference, proposals to change the name of a gene can evoke considerable emotion. The stakes, however, are high: with the correct identifier, a single search term can identify all genes in a gene family and in any groups of organisms. Except for chloroplast genes, the nomenclature of which were standardized some years ago[2,3], and plant mitochondrial genes, which were standardized more recently[4], such direct and comprehensive searches are currently impossible.

The advantages to scientific communication of having a common genetic language are obvious, and scientific journals play an indispensable role in publicizing nomenclature. Sometimes, however, and fortunately rarely, recommendations for gene nomenclature are circulated that have a limited application or short-lived utility and are, therefore, unsatisfactory. We have developed a comprehensive set of guidelines, and are in the process of extending mnemonics to all gene families. We expect that the simplicity of CPGN algorithms and the plant-wide commonality of the nomenclature will benefit and improve communications among scientists.

To forestall future ambiguities in the literature, and to avert the proliferation of multiple designations for the same gene, we need the help of the scientific community involved in relevant areas of research. We would request that authors preparing to submit research or review manuscripts in which they propose to name a gene or gene family first contact either of the CPGN offices. We will work with the authors to derive a satisfactory gene mnemonic that does not clash or conflict with those already assigned. We will attempt to do this as speedily as possible, to enable nomenclature to be established before manuscript submission. It would then be helpful for authors to indicate to the journal editor that the gene name has CPGN approval. This would publicize our efforts and help to promote adoption of CPGN nomenclature. The CPGN will ensure, on behalf of the authors, that the agreed gene mnemonic appears in the DNA and protein sequence databases.

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PII: S0167-7799(96)20010-1

Biotechnology and the red seaweed polysaccharide industry: status, needs and prospects

Donald Renn

Significant amounts of marine macroalgal (seaweed) polysaccharides are used in food, pharmaceuticals and other products for human consumption. Thus, the global seaweed polysaccharide industry operates in a highly regulated environment. Genetic manipulation of macroalgae to alter composition or growth characteristics may lead to products that do not fall within the current regulations: research that is readily translatable to industrial application is generally restricted to seaweed cultivation and processing and new applications of the approved polysaccharides. There is a great need, however, for research into the genome structure and metabolic pathways of commercially important marine macroalgae. This pre-competitive research may not be immediately applicable to the seaweed polysaccharide industry but is critical for sustaining future commercial growth.

Polysaccharides produced by marine macroalgae (seaweeds) form the basis of an economically important and expanding global industry[1,2]. Key products are agars[3,4], agaroses[3,5], algins[6,7] and carrageenans[8,9]. These are used as ingredients in food, pharmaceuticals and diverse consumer products and industrial processes (Box 1). Despite the potential for eventual commercial application of basic research into the biology of marine macroalgae, and subsequent alteration of biosynthetic pathways, the seaweed polysaccharide industries have been slow to accept changes and have, therefore, given minimal support for this research. Economic considerations are probably the main factor determining this attitude. The majority of commercial polysaccharide products have existed for many years with the same specifications. Companies are reluctant to alter those long-accepted extracts and blends and the raw materials from which they are recovered. The regulatory hurdles associated with the introduction of novel products or processes, coupled with an overriding need to minimize production costs in a competitive industry, mean that the majority of ongoing commercial research targets the development of alternative, less costly production methods and new applications for established products rather than more fundamental studies. This article considers the current status of basic research, focusing on progress in the application of molecular biology techniques to macroalgae. Integration of a better understanding of this molecular biology into current R&D strategies could enable translation of basic research into new products, processes or sources of raw materials.

Research and development on the marine macroalgae and the polysaccharides they produce may be considered in two categories: (1) basic and applied work supporting the immediate and short-term needs of the industries; and (2) long-term R&D.

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Short-term R&D

To reach the present sustainable, cost-effective status of seaweed cultivation, key areas of research have been: species and strain selection, vegetative reproduction, methods and locations for cost-effective cultivation, timing of and methods for harvesting, and optimizing drying and storage conditions. Processing the macroalgae to obtain the polysaccharides in appropriate forms for commercial utilization has evolved over many years with the origin of many key steps dating back over half a century. Research and development has been required to optimize the yields and assure product quality. In addition, process modifications have been, and continue to be, needed for matching product properties to new applications. This R&D has been reviewed previously[3,4,8,9].

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PII: S0167-7799(96)10069-X

Box 1. Commercially important seaweed polysaccharides

Although some marine macroalgae, or seaweeds, are eaten as food, particularly in the Asian-pacific region, and other biochemicals derived from them have small niche markets, their major economic value is associated with the polysaccharides certain species contain. Algins, carrageenans and agars have all achieved commercial significance because of their food and industrial applications and are the basis of an estimated billion dollar global effort[1,2]. This encompasses all segments of activity, from gathering wild harvests of the seaweeds, cultivating and collecting important varieties, processing, to preparing finished products containing the polysaccharides. Each of the major polysaccharides, as well as products derived from them (e.g. agaroses from agars), also has important life sciences applications[46,47]. For example, a number of the significant advances in modern biotechnology are based on the availability of various types of agarose. Immobilized enzyme reactors frequently rely on microorganisms encapsulated in calcium alginate or potassium κ-carrageenate gel. Insulin-producing pancreatic cells encapsulated in calcium alginate and implanted in patients' abdomens are keeping previously incapacitated diabetics alive and active. Carrageenans are used in medical research to induce a particular response in model systems and are being used in screening assays for new drugs. In particular, the rat-paw edema assay has led to the development of many of the currently used anti-inflammatory drugs.

The four major classes of marine macroalgae are Rhodophyta (red algae), Phaeophyta (brown algae), Chlorophyta (green algae) and Cyanophyta (blue–green algae). Only the red and brown macroalgae are currently sources of polysaccharides of significant commercial value. The seaweeds from which these polysaccharides are extracted are harvested from their natural growth, gathered from beaches or collected from cultivated beds. Carrageenans[8,9] and agars[3,4], from which agaroses[3,5] are derived by purification, are obtained from different genera of red algae. Algins[6,7] are obtained from a number of species of brown algae and are present in all. Tables 1 and 2 summarize the sources, compositions, properties and more important applications of these polysaccharides. Although a number of competitive gelling and viscosifying hydrocolloids exist, the unique properties of the seaweed polysaccharides are the sustaining force behind this industry.

Applications R&D is essential to develop new markets for seaweed polysaccharides. Changing consumer requirements and competitive markets drive R&D. One of the present driving forces is low-fat/low-calorie food products. Research directed towards elucidating the chemical composition of the various marine macroalgal polysaccharides has been important in seaweed selection and processing and finished product applications. Feeding and toxicology studies are assuring the safety of the ingested polysaccharides.

Table 1. Seaweed polysaccharides: sources and compositions

Polysaccharides	Important raw materials (genera)	Composition
Agars and agaroses	*Gelidium, Gracilaria, Pterocladia, Gracilariopsis, Porphyra*	Alternating 1,4-linked α-D-galactose and 3,6-anhydro-α-L-galactose backbone (agarobiose) substituted with varying percentages of methoxyl, ester sulfate and ketal pyruvate groups
Algins/alginates	*Macrocystis, Laminaria,* (Microbial: *Azotobacter vinelandii, Pseudomonas* sp.)	1,4-linked α-L-guluronic acid and β-D-mannuronic acid subunits in GG, MM and MG domains
Carrageenans		*kappa-* and *iota-* Alternating 1,3-linked α-D-galactose and 1,4-linked 3,6-anhydro-β-D-galactose backbone (carrabiose) substituted with varying percentages of ester sulfate
kappa-	*Eucheuma (cottonii), Kappaphycus (alvarezii), Gigartina (radula)*	4-sulfated on the galactose subunits (~25% ester sulfate)
iota-	*Eucheuma (spinosum)*	4-sulfated on the galactose subunits and 2-sulfated on the 3,6-anhydrogalactose subunits (~32% ester sulfate)
lambda-	*Chondrus (crispus), Gigartina (radula)*	Alternating 2-sulfated 1,3-linked α-D-galactose and 2,6-disulfated 1,4-linked β-D-galactose backbone (minimal 3,6-anhydro-β-D-galactose) (~35% ester sulfate)

Table 2. Seaweed polysaccharides: important properties and selected applications

Polysaccharides	Important properties	Selected applications
Agars	Gel aqueous solutions at low concentrations Form thermoreversible gels Relatively inert Significant degree of hysteresis Retain moisture Resist hydrolysis by terrestrial microorganisms	Baking icings Jelly candies Canned meats Dental impression media Laxatives Microbial culture matrix Raw material for agarose
Agaroses	Gel aqueous solutions at low concentrations Form ion-dependent thermoreversible gels Controllable electroendosinosis (EEO) Minimal non-specific protein reactivity Significant degree of hysteresis	Matrices for: Electrophoresis Immunoassays Microbial and cell culture Chromatography Immobilized systems
Algins/alginates	Ammonium and alkali metal salts are soluble in water, whereas free alginic acid and alkaline earth and Group III salts are insoluble and can form gels Bind water Thicken aqueous systems Suspend solids	Frozen foods to maintain structure on thawing Baking icings Salad dressings Tabletting agent Dental impression media Textile sizing Matrices for immobilized systems
Carrageenans	Bind moisture Stabilize emulsions Control flow and texture properties of food systems High protein reactivity – strong interactions with milk proteins	Frozen dessert stabilizers Chocolate milk stabilizer Texturizers for low-fat foods Low-calorie jellies Toothpaste binders Air-freshener gels Personal care products Pet foods
kappa-	Form strong rigid aqueous gels with potassium and calcium ions Exhibit synergy with locust bean and konjac gums	
iota-	Form elastic aqueous gels with calcium ions Exhibit synergy with locust bean gum and starch Suspend particulates	
lambda-	Non-gelling aqueous system viscosifier	

The development of analytical methods has been important in enabling the determination of molecular structures and classifying the polysaccharides produced by a given algal species, maintaining consistency of commercial polysaccharide products, and determining the fate of the polysaccharides in finished products. As appropriate analytical tools and techniques develop, they are being applied within the industry.

Significant variations in seaweed polysaccharide molecular structure can, and do, occur. The success of the industry has relied on both minimizing these variations for standardized products and exploiting them for new products.

Much of the above R&D has been supported within the marine algal polysaccharide industries and is surrounded by proprietary concerns. Pre-competitive base-building algal and algal polysaccharide research has, however, been given only token support by the industry, and then only as it meets near-term needs.

Long-term R&D

Although genetic manipulation of land plants by recombinant DNA (rDNA) technology is becoming routine, little attention has been focused on applying this approach to marine macroalgae. Seaweeds are also amenable to genetic manipulation, through either conventional crossbreeding or the emerging techniques of molecular biology. However, many seaweed polysaccharides are destined for human consumption, and are therefore strictly regulated. In some countries, including the USA, only carrageenans from specific seaweeds meet food-ingredient regulations, and this discourages the quest for new species as sources of raw materials. Without question, regulatory hurdles will become more numerous or stringent and act as a

greater deterrent to industry's pursuit of 'engineered' seaweed polysaccharides for human consumption. For non-regulated applications, cost will determine product success.

In addition to their use in establishing a more accurate taxonomy of marine macroalgae, advances in molecular and cell biology techniques will facilitate:

- mutation and mutant selection;
- crossbreeding through protoplast fusion to create hybrids;
- reduction of marine macroalgae to single cells and their *in vitro* culture;
- use of seaweeds as production hosts for heterologous expression;
- identification of, and transfer of, algal biosynthetic pathways for polysaccharides or other desired materials into prokaryotic production hosts;
- metabolic engineering of algae to enhance the production of, for example, polysaccharides;
- identification of algal genetic control elements (e.g. promoters).

Appropriate vectors, or techniques, must be identified that can be used to introduce foreign genes. High efficiency transformation methods must be developed. Finally, the regeneration of sustained growth and/or reproduction-capable algae from transformed protoplasts and cells needs to be developed as a routine technique.

Taxonomy using molecular biology

Perhaps the biggest barrier to acceptance of new sources for the production of marine macroalgal polysaccharides in the current, strict regulatory environment is the need to prove equivalence of raw materials. Many regulations were established well before analytical methods such as NMR spectroscopy were available, and plant morphology was the main criterion in taxonomical classification of marine macroalgae. Because the species acceptable as sources have been identified by name in the regulations, alternative, perhaps more abundant or more easily cultivated, species are often excluded even though polysaccharide extracts from them may be essentially identical. Conversely, existing taxonomic classification has not been infallible, and some seaweeds classified as closely related actually contain different polysaccharides. Ultimately a combination of molecular biology, refined analytical methods and traditional morphological taxonomy will produce a more appropriate list of acceptable raw materials. A recent paper[10] reporting on such an approach suggested a revision of systematics of one genera (Nizymeniaceae, Gigartinales, Rhodophyta) based on polysaccharide content, anatomy and nucleotide sequences.

The application of nucleic acid analysis to the taxonomy/algal classification problem has involved: (1) restriction enzyme fragment analysis; and (2) DNA sequence homologies. This research has enabled the creation of phylogenetic maps, and assisted the classification process.

However, anionic polysaccharides in marine macroalgae have similar properties to the nucleic acids (they are precipitated by ethanol at about the same pH as nucleic acids), and thus complicate isolation of the DNA and RNA. The presence of even minute quantities ($<1\ \mu g\ ml^{-1}$) of these can interfere with restriction enzyme digestions, and has necessitated development of modified DNA and RNA isolation methods[11–13].

Electrophoresis of plastid DNA fragments, obtained by digestion with restriction endonucleases, has been used to determine relatedness in the red marine macroalgae[14]; one study[15] used this technique to prove that two macroalgae previously thought to be distinct species were actually identical.

Targets for sequence-homology determination among the red seaweeds have been the 5S ribosomal RNA[16], nuclear small-subunit rRNA genes[17], the plastid *rbcL* gene coding for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo)[18], nuclear genes encoding cytosolic and chloroplast glyceraldehyde-3-phosphate dehydrogenases (GADPH)[19], gene amplification products[20], the β-tubulin gene[21], subunit 3 of cytochrome *c* oxidase[22], and small-subunit rRNA (SSU rRNA) from the mitochondria[23].

Nuclear DNA reassociation kinetics have also been used to determine inter- and intraspecific variations in selected agarophytes and carrageenophytes[24–27].

Protoplast formation, callus culture and plant regeneration

At present, obtaining sufficient amounts of selected strains of marine macroalgae for cultivation remains a problem in that current methods require taking up to 25% of the cultivated material as the seed. This reduces productivity and, in cultivation efforts where the seedstock is attached to the supporting structure by hand, it is labor intensive. The ability to obtain viable macroalgal protoplasts that can divide, form cell walls and regenerate into whole plants is critical to a number of important processes, including the production of hybrid and transgenic macroalgae. If generation of viable protoplasts that form cell walls and divide could be achieved reproducibly and on a large scale, the protoplasts could be used as seedstock for macroalgal culture. Significant progress in this direction has been reported for one commercially important seaweed, *Porphyra linearis*[28]. Success in obtaining viable protoplasts has been reported for *Gracilaria tikvahiae*[29], other strains of *Porphyra*[30], *Chondrus crispus*[31] (followed by cell wall regeneration and division), *Gracilaria verrucosa*[32] and *Kappaphycus alvarezii*[33]. The paper on *K. alvarezii* also reports that the protoplasts secreted carrageenan fragments.

Progress has also been made in the field of callus, or undifferentiated cell, culture of marine macroalgae. Success has been reported for various *Gelidium* and *Gracilaria* agarophytes[34] and *Pterocladia capillacea*[35]. Under appropriate conditions, seaweed polysaccharides can be produced by callus culture, including agar from *P. capillacea*[35]. Whether this could ever be a

cost-effective production method for polysaccharides is doubtful. However, it is another way of providing cultivation seedstock and material for biosynthetic pathway determinations.

Gene mapping and sequencing

A prerequisite for either genetic or metabolic engineering is an adequate understanding of genome structure, sequence and gene expression. Although the homology is not complete, probes from land plants, unicellular algae and other microorganisms have been used with success to locate specific genes. Considerable groundwork has been done using unicellular red and green algae, and cyanobacteria, but reports of mapping and sequencing red marine macroalgal genomes are sparse. The chloroplast ribosomal-protein-encoding genes of the agarophyte *Gracilaria tenuistipitata* have been located, cloned and characterized[36]. A 1365 bp region around this gene was also sequenced; the gene order was found to be identical to that detected in the chloroplast DNA of liverwort, tobacco and maize. The plastid gene for the rp122 protein in *G. tenuistipitata* has also been isolated and sequenced[37].

The polymerase chain reaction (PCR) works with seaweed DNA and has been used to amplify *Gracilaria pacifica* nuclear and plastid ribosomal genes from algal herbarium specimens and algal spores[38]. The glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene system of the red alga *C. crispus* has been investigated[39]. Promoter structures, intron/exon organization, genomic complexity, differential expression of genes and transcript level of the genes in gametophytes and protoplasts were determined, paving the way for genetic transformations in *Chondrus*.

Transformation, protoplast fusion

Methods for the introduction of foreign genes into other organisms include protoplast fusion; introduction of the desired gene(s) by appropriate vectors, including insertion of transformed plasmids; infection with transformed viruses or other specific pathogens; and direct insertion of the gene by, for example, electroporation or ballistics.

In order to use the transformed plasmid technique, plasmids must be present in the host. Five of 21 red algal genera studied[40] were found to contain circular dsDNA plasmids. Some of these were isolated and characterized, and one 3.5 kbp plasmid from *Gracilariopsis lemaneiformis* was sequenced to reveal two potential open reading frames. In this species, plasmids are present in a high copy-number per cell and may provide useful vectors for algal transformations. The DNA sequence and structural organization of the GC2 plasmid from the agarophyte *Gracilaria chilensis* has been determined[41]. This 3827 bp circular plasmid has one major open reading frame that generates a transcript and could encode a 411 amino acid polypeptide.

In attempting to determine whether protoplast fusion techniques could be used for algal transformation, factors affecting the electrofusion efficiency of *Porphyra yezoensis* protoplasts were studied[42]. Fusion efficiencies were found to depend on the concentration and nature of reagents used to adjust the osmotic pressure of the medium. A recent paper[43] suggests that recombinant viruses, particularly the large dsDNA viruses that are known to infect eukaryotic algae, can be used as transformation vectors for marine macroalgae.

Impact of current research on commercial concerns

To sustain growth in a competitive environment, the seaweed polysaccharide industries devote significant effort to reducing production costs and to finding new applications for polysaccharides. Most research supported by industry, both internally and externally, is therefore focused on short-term needs. However, ongoing basic-research efforts to classify marine macroalgae by the chemical structure of the polysaccharides they produce and their relatedness as determined by genomes, and to develop new strains and improve seaweed culture methods, are also directly relevant to industry. The first of these, in particular, is an important step towards gaining regulatory acceptance of a wider range of source species.

Long-range research into elucidating the biosynthetic pathways for polysaccharide(s) production, and the development of methods for genetic manipulation, are prerequisites to engineering macroalgae capable of synthesizing, for instance, polysaccharides enzymatically processed by the algae so that only aqueous extraction and recovery or washing and grinding are needed to prepare commercially acceptable products. Such technological advances are also essential for the development of seaweeds as heterologous production hosts. If mammalian antibodies can be produced in tobacco plants[44] and the rabies virus glycoprotein in tomatoes[45], why not value-added products in seaweeds? Macroalgae may be a cheaper alternative 'host' to produce these products.

Overcoming existing barriers

Many, but not all, of the barriers to acceptance of new marine macroalgal raw material and new or altered polysaccharides are regulatory. Probably the single most important advance would be to base regulations on analytical data for polysaccharide(s) that are present rather than rely on the outmoded classical seaweed taxonomy. Not only would this open up new supply channels for basic raw materials for industry, but by doing so would create economic opportunities in some developing countries.

Further into the future, products from genetically altered algae need to be considered: they will present the same regulatory problems that surround genetically engineered land plants whose products are targeted for human consumption. The main consideration for polysaccharides from these algae will probably, however, be whether they offer a cost-effective advantage. A parallel situation may be plants such as maize or potatoes engineered for altered starch

composition. There may be sufficient applications for engineered polysaccharides in the non-consumable markets to warrant the cost of producing them.

Pre-competitive research is slowed by the low level of funding. Inevitably, researchers focus on areas with the most abundant support, and more funding is needed if the field is to advance at a rate more in line with its potential importance.

Future prospects

Polysaccharides from marine macroalgae are the basis of well-established, growing industries. Many consumer products rely on their unique properties and would not exist without their availability. Important advances in modern medicine and biotechnology would not have been possible without their existence[46,47]. With increased research emphasis, new applications and possibly even new seaweed polysaccharides will emerge. Genetic understanding and techniques for the insertion of foreign genes are evolving. Overall, the future looks bright for marine macroalgae and the polysaccharides they produce. However, to sustain continued growth, the industries involved must support scientists working to develop the pre-competitive research base upon which future, commercially important discoveries will be made.

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Environmental stress response in plants: the role of mitogen-activated protein kinases

Tsuyoshi Mizoguchi, Kazuya Ichimura and Kazuo Shinozaki

Mitogen-activated protein kinase (MAPK) cascades have essential roles in diverse intracellular signaling processes in plants, animals and yeasts. In plants, transcription of genes encoding protein kinases involved in MAPK cascades is upregulated by environmental stresses and plant hormones; in addition, MAPK-like kinase activities are transiently activated in response to environmental stresses. Consequently, MAPK cascades are now thought to have important roles in stress signal transduction pathways in higher plants.

Mitogen-activated protein kinases (MAPKs) were initially identified as serine/threonine kinases that were activated by various growth factors and in phosphorylation cascade reactions occurring during mitosis in animals[1–3]. MAPKs have key roles in integrating multiple intracellular signals transmitted by various second messengers. MAPK cascades are composed of three protein kinases: MAPKs, MAPK kinases (MAPKKs), and MAPKK kinases (MAPKKKs) (Fig. 1). MAPKs are activated when both tyrosine and threonine residues in the TXY motif are phosphorylated by MAPKKs. MAPKKs are activated when serine and serine/threonine residues in the SxxxS/T motif are phosphorylated by MAPKKKs. MAPK cascades have been shown to function in various signal transduction pathways in animals and yeasts[1–5] (Fig. 2). In plants, many cDNAs for MAPKs, MAPKKs and MAPKKKs have been isolated from various species. A larger number of MAPKs have been identified in plants than in animals and yeasts, which suggests that MAPK cascades have a wider variety of roles in plants. However, their functions are not yet clear. Recently, it has been demonstrated that both MAPK-like kinase activity and mRNA levels of the components of MAPK cascades increase in response to environmental stress in plants. This suggests that some of the plant MAPK cascades have important roles in environmental stress responses and plant hormone signal transduction.

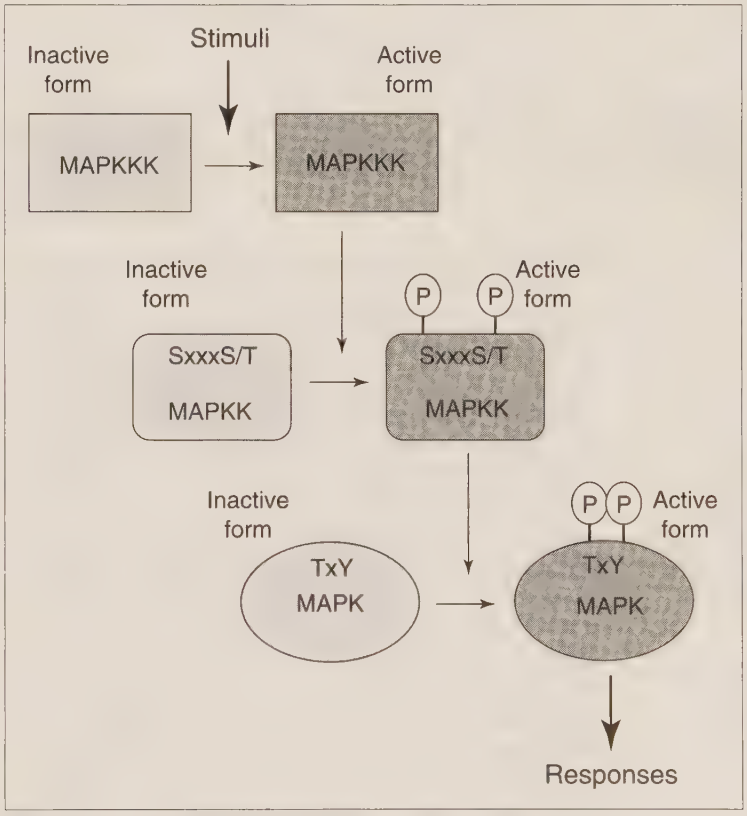

Figure 1
Regulation of the mitogen-activated protein kinase (MAPK) cascade by phosphorylation. An inactive, dephosphorylated MAPK is activated by phosphorylation of both threonine and tyrosine residues in the sequence TxY, which is catalyzed by an immediate upstream MAPK kinase (MAPKK). An inactive, dephosphorylated MAPKK is activated by phosphorylation of both serine and serine/threonine residues in the SxxxS/T sequence, which is catalyzed by an immediate upstream MAPKK kinase (MAPKKK).

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PII: S0167-7799(96)10074-3

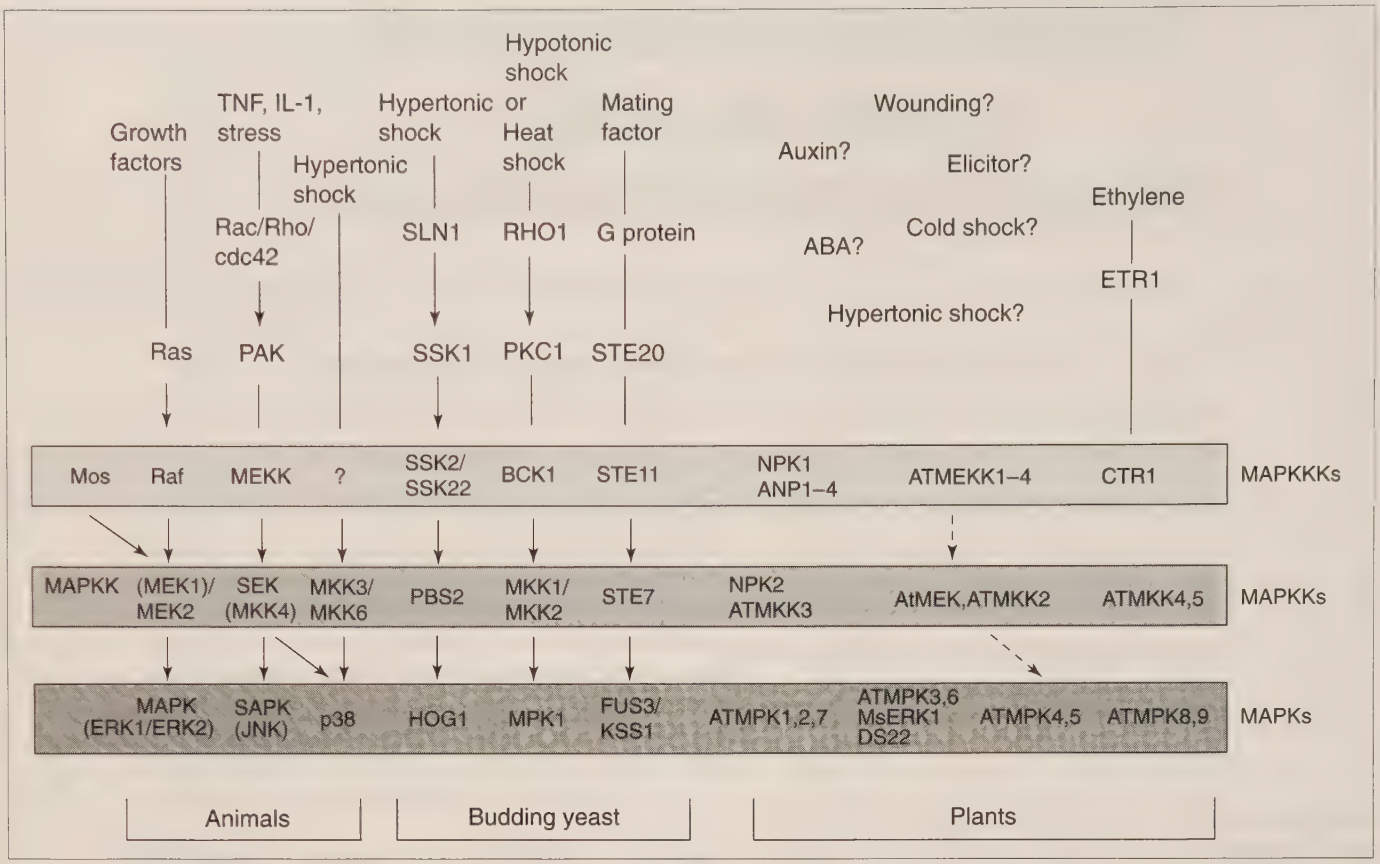

Figure 2

A hypothetical model for mitogen-activated protein kinase (MAPK) cascades in various signal transduction pathways in animals, yeasts and plants. There are at least four MAPK cascades in animals and at least five MAPK cascades in *Saccharomyces cerevisiae*. Numerous MAPKKK/MAPKK/MAPK homologs have been identified in plants. Plant MAPKs are classified into four subgroups. See text for details.

MAPK cascades and stress responses in animals

In animal cells, at least four different MAPK cascades function in the different signaling pathways (Fig. 2). A cascade involving Raf/Mos, MEK1/MEK2 and ERK1 operates in growth-factor-dependent cell proliferation. The response to stresses such as UV irradiation, translational inhibitors, heat shock and tumor necrosis factor involves a cascade comprising MEKK, SEK/MKK4 and SAPK/JNK, whereas environmental stresses such as osmotic shock and UV irradiation involve the cascade MKK3/MKK6/MKK4 and p38 (Refs 1–3,6). Recently, a new MAPKK homolog, MEK5, was isolated by polymerase chain reaction (PCR), and a new MAPK homolog, ERK5, was identified by a specific interaction with MEK5 in the yeast two-hybrid system[7]. The MEK5–ERK5 pathway is the fourth candidate of the animal MAPK cascades. The animal MAPKs, ERK1, ERK2 and ERK5 all share the conserved phosphorylation site motif TEY (Refs 1–3,7). By contrast, SAPK/JNK and p38 have the motif TPY and TGY, respectively[1–3,6].

MAPK cascades and stress responses in yeasts

In *Saccharomyces cerevisiae*, at least five different MAPK signaling pathways have been described[1–3]: (1) the mating-pheromone-response pathway (STE11, STE7 and FUS3/KSS1); (2) the protein kinase C (PKC)-dependent signaling pathway, which has a role in maintenance of cell wall integrity (BCK1, MKK1/MKK2 and MPK1); (3) the osmoregulatory pathway (SSK2/SSK22, PBS2 and HOG1); (4) the pseudohyphal differentiation pathway (STE11 and STE7); and (5) the spore wall assembly pathway (SMK1). In *Schizosaccharomyces pombe*, at least two different MAPK signaling pathways have been described[1–3,8]: the mating-pheromone-response pathway (byr2, byr1 and spk1); and the osmoregulatory pathway (wis1 and sty1). The yeast MAPKs FUS3, KSS1, MPK1 and spk1 have the motif TEY at the phosphorylation site[1–3], whereas HOG1 and sty1 have the motif TGY, and SMK1 has the motif TNY (Refs 1–3,8).

When yeast cells are exposed to high osmolarity, glycerol synthesis is induced to increase internal osmolarity and the MAPK cascade involving SSK2/SSK22, PBS2 and HOG1 is activated. Maeda *et al.* have identified new eukaryotic components (SLN1 and SSK1) that function as direct upstream factors of this MAPK cascade[1–3]. In the 'two-component regulatory system' SLN1p and SSK1p have high sequence identity to the sensor and regulator factors, respectively. Two-component regulatory systems are widespread in bacteria and function in various signal transduction pathways. Maeda *et al.* also demonstrated that SHO1p directly interacts with and activates PBS2p in a different

pathway from the Sln1–Ssk1–Ssk2/Ssk22 pathway[9]. Recently, Davenport *et al.* have shown that the BCK1–MKK1/MKK2–MPK1 pathway functions in the hypotonic shock response[10]. These observations indicate that the MAPK cascades in yeasts have important roles not only in mating and developmental signaling processes but also in mediating cellular responses to environmental stresses.

Plant homologs of protein kinases in MAPK cascades

A number of genes for MAPKs have been reported in higher plants[4,5,11–21] (Table 1). We have demonstrated in *Arabidopsis thaliana* that MAPKs constitute a gene family with at least nine members (ATMPK1–9), which can be classified into four subgroups based on phylogenetic analysis of their amino acid sequences[11,12] (Mizoguchi and Shinozaki, unpublished). ATMPK8 and ATMPK9 are classified into the fourth new subgroup. Instead of the motif TEY on animal ERKs, ATMPK8 and ATMPK9 contain the sequence TDY (Mizoguchi and Shinozaki, unpublished).

NPK2, which is structurally related to MAPKK, has been isolated from tobacco[5,22]; NPK2-related genes have been revealed in various plant species by Southern blot analysis, and two types of *Arabidopsis* cDNA clones that hybridize with NPK2 have been isolated[5]. Recently, Morris *et al.* have identified one, AtMEK (P. Morris and J. Giraudat, pers. commun.), and we have identified four additional *Arabidopsis* cDNAs, ATMKK2–4 (Ichimura, Mizoguchi and Shinozaki, unpublished), encoding protein kinases that have significant homology to MAPKKs. These plant MAPKK homologs can be classified into three subgroups based on phylogenetic analysis of their amino acid sequences (Ichimura, Mizoguchi and Shinozaki, unpublished).

Three types of MAPKKK homologs have been isolated from plants. The CTR1 gene, encoding a Raf homolog that functions as an activator of MAPKK in animal cells[1–3], has a key role in the ethylene signal transduction pathway[23]. NPK1 (Ref. 24), which is structurally related to MAPKKKs, has been isolated from tobacco and shown to complement the growth defect of the yeast *bck1*Δ mutant. Nishihama *et al.* have isolated three cDNAs (ANP1–3) from *Arabidopsis* that encode homologs of NPK1 (Ref. 5). We isolated *Arabidopsis* cDNAs (ATMEKK1–4) encoding protein kinases that are structurally related to MAPKKKs (such as Byr2, STE11, BCK1, MEKK and NPK1)[25]. ATMEKK1 can complement the yeast *ste11* mutant in response to the mating pheromone[25].

Table 1. Summary of the isolated cDNAs that encode MAPK, MAPKK and MAPKKK homologs in plants

Gene name	Plant species	Group[a]	Refs
MAPKs			
ATMPK1	*Arabidopsis thaliana*	1	11
ATMPK2	*Arabidopsis thaliana*	1	11
ATMPK7	*Arabidopsis thaliana*	1	12
NTF3	*Nicotiana tabacum*	1	13
PMEK1	*Petunia hybrida*	1	14
ATMPK3	*Arabidopsis thaliana*	2	12, 25
ATMPK6	*Arabidopsis thaliana*	2	12
MsERK1 (MsK7)	*Medicago sativa*	2	15, 16
D5	*Pisum sativum*	2	17
Asmap1	*Avena sativa*	2	18
NTF4	*Nicotiana tabacum*	2	19
NTF6	*Nicotiana tabacum*	2	19
MMK1	*Medicago varia*	2	20
DS22	*Nicotiana tabacum*	2	21
ATMPK4	*Arabidopsis thaliana*	3	12
ATMPK5	*Arabidopsis thaliana*	3	12
MMK2	*Medicago varia*	3	20
ATMPK8	*Arabidopsis thaliana*	4	b
ATMPK9	*Arabidopsis thaliana*	4	b
MAPKKs			
NPK2	*Nicotiana tabacum*	1	22
ATMKK3	*Arabidopsis thaliana*	1	c
AtMEK	*Arabidopsis thaliana*	2	d
ATMKK2	*Arabidopsis thaliana*	2	c
ATMKK4	*Arabidopsis thaliana*	3	c
ATMKK5	*Arabidopsis thaliana*	3	c
MAPKKKs			
NPK1	*Nicotiana tabacum*	1	24
ANP1	*Arabidopsis thaliana*	1	5
ANP2	*Arabidopsis thaliana*	1	5
ANP3	*Arabidopsis thaliana*	1	5
ATMEKK1	*Arabidopsis thaliana*	2	25
ATMEKK2	*Arabidopsis thaliana*	2	25
ATMEKK3	*Arabidopsis thaliana*	2	25
ATMEKK4	*Arabidopsis thaliana*	2	25
CTR1	*Arabidopsis thaliana*	3	23

[a]MAPK, MAPKK and MAPKKK homologs in plants can be classified into four, three and three subgroups, respectively, based on our phylogenetic analyses.
[b]Mizoguchi *et al.*, unpublished.
[c]Ichimura *et al.*, unpublished.
[d]P. Morris *et al.*, unpublished.

Interactions of the components of MAPK cascades in higher plants

A number of genes for MAPKs and several genes for MAPKKs and MAPKKKs have been reported in higher plants[4,5,11–25]. However, there have been no reports of direct interactions between MAPK and MAPKK or between MAPKK and MAPKKK from plants, and no evidence of direct phosphorylation and activation either of MAPK by MAPKK or of MAPKK by MAPKKK from plants. Recently, we demonstrated that ATMPK4 specifically interacts with AtMEK in the yeast two-hybrid system (Mizoguchi, Morris and Shinozaki, unpublished). Furthermore, coexpression of ATMPK4 with AtMEK suppressed the *mpk1*Δ ($MAPK^-$) and the *bck1*Δ ($MAPKKK^-$) yeast mutations (Mizoguchi, Morris and Shinozaki, unpublished). These results suggest that AtMEK (MAPKK) functions as a direct upstream factor of ATMPK4 (MAPK). By using the yeast two-hybrid system and complementation analysis of yeast mutants we have also found

that ATMEKK1 (MAPKKK) not only interacts with but also activates AtMEK (MAPKK) (Mizoguchi, Morris and Shinozaki, unpublished). These observations suggest that ATMEKK1, AtMEK and ATMPK4 constitute a MAPK cascade in *Arabidopsis* cells.

MAPK-like activities in plants

Plants have MAPK-like kinase activities with similar biochemical characteristics to those of known MAPKs of yeast and animal cells. Myelin basic protein (MBP) is a good substrate for MAPKs, such as rat ERK1/ERK2 (Refs 1-3), budding yeast Mpk1 (Refs 1–3), alfalfa MsERK1 (Refs 13,14) and *Arabidopsis* ATMPK1/ATMPK2 (Ref. 11). We demonstrated that a 46 kDa protein kinase that phosphorylates MBP was rapidly and transiently activated by the plant hormone auxin analog 2,4-dichlorophenoxyacetic acid (2,4-D) in auxin-starved tobacco BY-2 cells[11]. Protein kinase activities that phosphorylated recombinant ATMPK2 protein were also increased rapidly by 2,4-D treatment in auxin-starved cells[11]. Knetsch *et al.* reported that the plant hormone abscisic acid (ABA) induced a rapid and transient activation of a MAPK-like activity in barley aleurone protoplasts[26]. Suzuki *et al.* also detected a transient activation of a 47 kDa MBP kinase activity in cultured tobacco cells treated with fungus-derived elicitors[27]. This protein kinase is phosphorylated on tyrosine residues, and activated by upstream protein kinase(s) in cultured tobacco cells[27]. Usami *et al.* demonstrated that a 46 kDa protein kinase that phosphorylated MBP was rapidly and transiently activated in the wounded tissues of tobacco[28]. This MBP kinase required the phosphorylation of both tyrosine and threonine/serine residues for its activity. Furthermore, similar protein kinase activities were detected in various plant species[5]. However, to determine if these kinases are MAPKs, the peptide sequences, or biochemical analysis using specific antibodies against the cloned MAPKs, are required. Recently, Bögre *et al.* and Jonak *et al.* used a specific antibody against the alfalfa MAPK MMK4 to demonstrate that it is activated by a variety of stresses including mechanical stimuli, low temperature and drought[29,30].

Transcriptional control of the putative components of plant MAPK cascades in response to environmental stresses

We have demonstrated that the levels of transcripts for three protein kinases, ATMEKK1 (MAPKKK), ATMPK3 (MAPK) and ATPK19 (ribosomal S6 kinase or RSK homolog), increased markedly and simultaneously when plants were treated with touch stimuli, low temperature and salinity stress[12,25]. These results suggest that some of the MAPK cascades in plants function in transducing signals in the presence of environmental stress and that MAPK cascades are regulated at the transcriptional and post-translational level in plants[25]. Recently, Seo *et al.* used differential hybridization to isolate a tobacco cDNA clone (DS22) for a wound-inducible gene encoding a MAPK homolog (WIPK; Ref. 21). The transcript for WIPK increased rapidly after mechanical wounding. Analysis of WIPK overexpression in transgenic tobacco suggested that WIPK mediates jasmonic acid (JA)- and salicylic acid (SA)-induced wound signal transduction pathways. In the transgenic tobacco, the endogenous WIPK gene was silenced and the production of wound-induced JA was inhibited, followed by suppressed accumulation of wound-inducible gene transcripts. By contrast, the levels of SA and transcripts of SA-inducible, pathogenesis-related proteins increased abnormally upon wounding. The transcript level and kinase activity of MMK4 increased after cold and drought treatments of alfalfa plants[30]. Three MAPKs from different plant species, ATMPK3, WIPK and MMK4, have a higher sequence identity to one another than to other MAPKs, and the mRNA levels of these MAPKs increased after stress treatments, suggesting that these MAPKs play similar roles in plants under a variety of stress conditions. Wilkinson *et al.* have isolated a gene that co-segregated with the *Never-ripe* (*Nr*) locus[31], which encodes a protein with homology to the *Arabidopsis* ethylene receptor ETR1 (Ref. 32) and prokaryotic two-component signal transducers (histidine kinases). The NR mRNA is positively regulated by ethylene during fruit development. In *Arabidopsis*, the two-component histidine kinase ETR1 is thought to function upstream from the Raf homolog CTR1 in the ethylene signaling pathway[23,32]. In higher plants, the mRNAs of various genes involved in signal transduction pathways, such as protein kinases, a phospholipase C, a histidine kinase, monomeric small GTP-binding proteins, calmodulins and transcription factors, accumulate in response to environmental stimuli or stresses[33]. Under environmental stress conditions, the elevated levels of the mRNAs of these putative signal transducers may increase their protein level, which is likely to amplify the signal transduction efficiency of the cascade. Analyses of transgenic plants that overexpress or repress genes for MAPKs, MAPKKs and MAPKKKs will help to elucidate the molecular mechanisms of the stress response and adaptation to environmental changes in higher plants.

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Bacterial and archaeal S-layer proteins: structure–function relationships and their biotechnological applications

Uwe B. Sleytr and Margit Sára

Crystalline cell surface layers (S-layers) composed of planar assemblies of protein or glycoprotein subunits are one of the most commonly observed cell envelope structures of bacteria and archaea. Isolated S-layer subunits of numerous organisms are able to assemble into monomolecular arrays either in suspension, at liquid–surface interfaces, including lipid films, on liposomes and on solid supports. Pores in S-layers are of regular size and morphology, and functional groups on the protein lattices are aligned in well-defined positions and orientations. These characteristic features of S-layers have led to various applications in biotechnology, vaccine development, diagnostics, biomimetics and molecular nanotechnology.

In the course of evolution, prokaryotic organisms developed a broad spectrum of cell envelope structures[1]. Despite this considerable diversity, among the most commonly observed cell surface structures are two-dimensional arrays of proteinaceous subunits termed S-layers[2]. S-layers have been found on organisms of almost every taxonomic group of walled bacteria and represent an almost universal feature of archaea[3,4]. Most crystalline arrays are composed of a single protein or glycoprotein species with a molecular mass ranging from 40 000 to 200 000 (Refs 4,5) and cover the cell surface completely. A particularly useful electron microscopic preparation technique for identifying S-layers on a specific organism is freeze-etching of intact cells (Fig. 1a). High-resolution electron microscopic studies and scanning force microscopy in combination with computer image enhancement procedures have provided structural information on the mass distribution of S-layer lattices down to approximately 1.5 nm (Refs 4,6). S-layers exhibit either oblique (p1, p2), square (p4) or hexagonal (p3, p6) lattice symmetry with center-to-center spacing of the morphological units of 2.5–35 nm (Fig. 1b). Depending on the lattice type, a single morphological unit is composed of one, two, three, four or six identical subunits (Fig. 1b). Most S-layers are 5–15 nm thick and possess a fairly smooth outer surface and a more corrugated inner surface[3,4,6]. Structural studies have also revealed that S-layers are very porous protein meshworks with pores occupying 30–70% of their surface area. The monomolecular assemblies of identical subunits exhibit pores of identical size and morphology in the 2–8 nm range (Fig. 1c). In many S-layers, two, or even more, distinct classes of pores could be observed. In many species of bacteria, individual strains exhibit great diversity with respect to lattice symmetry and center-to-center spacing of the morphological units. In some organisms, two, or more, superimposed S-layer lattices composed of different S-layer proteins have been identified[4]. These structural data, including chemical studies and homology comparisons of protein sequences (Ref. 7), indicate that, at least in bacteria, S-layers are non-conserved structures and are of limited taxonomical value. More recently it was shown that even individual strains are capable of synthesizing different S-layer proteins[8–10].

Comparison of the primary structure of S-layer proteins and the genes encoding them from a broad spectrum of organisms has revealed a similar overall composition[5,11,12]. Most S-layers are composed of weakly acidic proteins (or glycoproteins) with isoelectric points between pH 3 and 6. S-layer proteins usually have a high amount of glutamic and aspartic acid (together approximately 15 mol%) but no, or only a few, sulfur-containing amino acids. Circular dichroism

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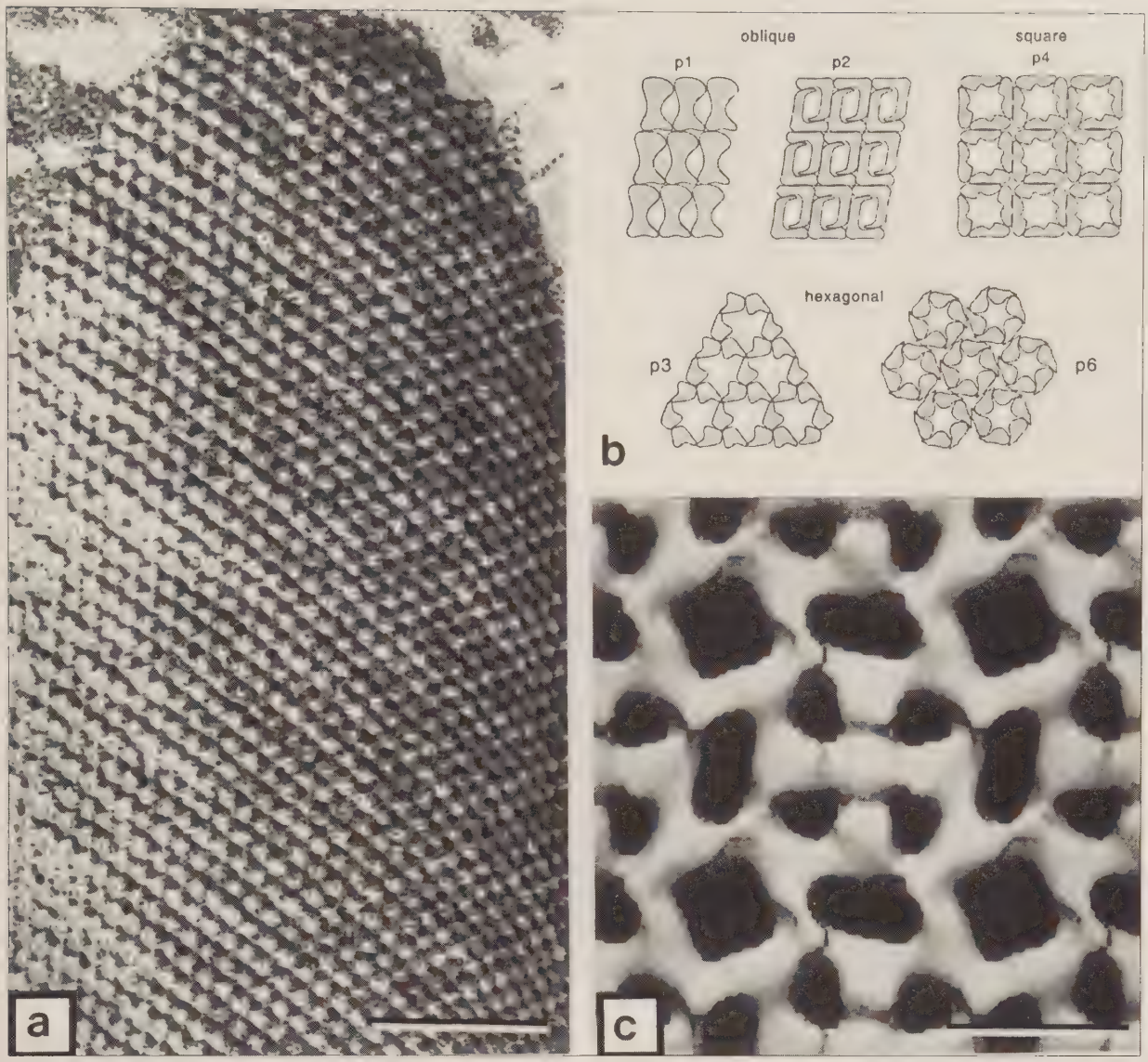

Figure 1

(**a**) Freeze-etching preparation of whole cells of *Thermoanaerobacter thermohydrosulfuricus*, showing a hexagonally ordered S-layer lattice. (**b**) Schematic drawing of different S-layer lattice types detected on prokaryotes. (**c**) Three-dimensional model of the square S-layer lattice of the strain variant *Bacillus stearothermophilus* NRS 2004/3a viewed on the outer surface. Scale bars: (**a**) 100 nm; (**c**) 10 nm.

measurements and amino acid sequence analyses show that typically about 40% of the amino acid residues are organized as β-sheet and 20% as α-helix structures[7]. An interesting feature of many archaea and selected bacteria is their ability to glycosylate S-layer proteins[11,13,14]. These carbohydrate moieties strongly resemble O-antigen polysaccharides of Gram-negative bacteria. They are polymers of linear or branched repeating sequences of two to six monosaccharide units and include a wide range of hexoses, deoxy and amino sugars, uronic acids or even sulfate and phosphate residues. The chain length may vary from a few sugars to approximately 150 monosaccharide residues. The glycan chains can be linked to the protein moiety by N- or O-glycosidic linkages. Different novel carbohydrate–amino acid linkage types have been detected[11,14] (e.g. asparaginyl–rhamnose, tyrosine–galactose).

Although S-layers represent an important class of secreted proteins, only about 30 S-layer genes have yet been cloned and sequenced. There is little information available on the regulation of S-layer protein synthesis, on the translocation of S-layer subunits across components of the cell envelope and about the S-protein domains involved in inter- and intramolecular interactions. This lack of knowledge is due mainly to the difficulties encountered in cloning S-layer genes in a form in which they are stably expressed at high levels[7].

The individual subunits of S-layers interact with each other and with the supporting envelope layer through non-covalent forces[4,6,15,16]. In Gram-positive bacteria, the S-layers are associated with the peptidoglycan-containing layer and in Gram-negative bacteria with components of the outer membrane. In archaea, which lack a rigid cell wall layer, S-layers are the only wall component external to the cytoplasmic membrane.

S-layers can usually be disrupted by chemical agents. Generally, a complete disintegration of S-layers from Gram-positive bacteria into constituent subunits can be obtained by treatment with high concentrations of agents (e.g. guanidinium hydrochloride) that break hydrogen bonds. Detailed studies have been performed on a variety of Gram-positive bacteria to elucidate the dynamic process of S-layer assembly *in vitro* and *in vivo*[15]. Isolated S-layer subunits have shown the ability to recrystallize into two-dimensional arrays in suspension or on suitable surfaces or interfaces after removal of the agent used for their isolation. In suspension, this entropy-driven recrystallization process can lead to self-assembly products with the morphology of flat sheets, open-ended cylinders or closed vesicles. The most detailed assembly studies were performed with S-layers of different Bacillaceae[15,17,18]. Differences in surface net charge and hydrophobicity of the inner and outer surface and defined intersubunit bonding properties are responsible for the proper orientation and incorporation of the S-layer subunits on cell surfaces. Being self-assembly systems, S-layers have the intrinsic ability to assume a closed structure with low free-energy during cell growth[15].

Provided that other cell-surface components (e.g. capsules and sheaths) are absent, S-layers, as the outermost envelope structure, represent an important interface between the cell and its environment. It is also evident that S-layers are generally part of complex supramolecular envelope structures and consequently should not be considered in functional terms as an isolated cellular component. Since prokaryotes carrying S-layers are ubiquitous in the biosphere and inhabit the most diverse ecological niches, it is not surprising that they can fulfil a broad spectrum of functions. Although many of the functions assigned to S-layers are still hypothetical, it is now recognized that these structures can provide the organism with a selection advantage by functioning as: (1) frameworks to determine cell shape and to aid in the cell division process in those archaea that possess S-layers as an exclusive wall component; (2) structures involved in cell adhesion and surface recognition; and (3) protective coats, molecular sieves, and molecule and ion traps. S-layers can contribute to virulence when present on pathogenic bacteria. Surveys of S-layer function are given in Refs 2,5,12,16,19–22.

Application potential of S-layers

S-layers have numerous potential applications in biotechnology and nanotechnology (see Box 1).

S-layers as isoporous ultrafiltration membranes

Analysis of the mass distribution and permeability properties of isolated S-layer lattices of various Bacillaceae revealed that they function as molecular sieves in the ultrafiltration range[23]. By contrast to conventional ultrafiltration membranes (which are made of amorphous polymers and reveal a wide pore-size distribution with pores differing by as much as one order of magnitude), S-layer ultrafiltration membranes (SUMs) are isoporous structures with very sharp molecular exclusion limits. SUMs are manufactured by depositing either S-layer-carrying cell wall fragments or S-layer self-assembly products on commercial microfiltration membranes with a pore size up to 1 μm in a pressure-dependent process[23,24].

The mechanical and chemical resistance of these composite structures is obtained by introducing inter- and intramolecular covalent linkages (e.g. with glutaraldehyde) between the individual S-layer subunits. Moreover, since S-layers are periodic structures composed of identical subunits, functional groups on the polypeptide chains (carboxyl, amino or hydroxyl groups) are located on each constituent subunit in an identical position and orientation. This uniformity of reactive groups on both the surface of the S-layer lattice and within the pores provides possibilities for a broad spectrum of chemical modifications to obtain differently charged or hydrophilic and hydrophobic ultrafiltration membranes with defined nominal molecular mass cut-offs[25,26]. By using SUMs with both defined pore size and physicochemical surface properties, it was possible for the first time to determine correlations between the pore size and the net charge of an ultrafiltration layer, the molecular characteristics (dimension, net charge) of adsorbed proteins, and flux losses caused by adsorption to the surface of the ultrafiltration layer[27]. This is an essential requirement for producing ultrafiltration membranes with tailored molecular sieving and antifouling characteristics.

S-layers as matrices for the immobilization of functional molecules

Because S-layer lattices possess identical physicochemical properties on each molecular unit at the subnanometer scale, they represent unique structures for a defined binding of differently sized functional molecules[27–29]. With glycosylated S-layers, not only the protein moiety but also the carbohydrate residues are available for immobilization of various macromolecules[29]. High-resolution electron microscopic studies revealed that molecules immobilized on either the protein or the carbohydrate moieties are frequently regularly arranged in a monolayer, reflecting the periodicity of the supporting lattice (Fig. 2)[28,29]. S-layer lattices can be used in different ways as immobilization matrices. Depending on the demands, either SUMs, S-layer self-assembly products (SAPs) or S-layer microparticles (SMPs) can be used[28,29]. SMPs are derived from different Gram-positive bacterial cells which are cleaved by ultrasonication. They represent cup-shaped structures, 1 μm in diameter, that are covered with an S-layer on both surfaces of the peptidoglycan-containing layer. The possibility of immobilizing functional molecules on different types of S-layer materials has led to a broad spectrum of actual applications, such as bioanalytical mono- and multi-enzyme sensors, including optodes[30,31], enzyme and affinity membranes, immunoassays, dipsticks and affinity microparticles[28,29,32].

The advantage of S-layers as a matrix for immobilization in comparison with the commonly used porous supports made of amorphous polymers can be seen in the extremely low unspecific adsorption of molecules to S-layers at ~pH7, which prevents background reactions. Moreover, the formation of monolayers of enzymes, ligands or antibodies on the S-layer surface means free-diffusion-controlled reactions can be avoided. SMPs with a densely packed monomolecular layer of Protein A were capable of adsorbing IgG from different solutions, including serum or hybridoma cell culture supernatants, up to the theoretical saturation capacity. These SMPs were able to withstand high centrifugation, shear forces and very harsh cross-flow conditions[33].

S-layers for vaccine development

Particular attention has been paid to S-layers present on the cell envelopes of pathogenic organisms. Detailed studies on the fish pathogenic bacteria *Aeromonas salmonicida* and *A. hydrophila*[34] and the human pathogen *Campylobacter fetus*, sub sp. *fetus*[35], revealed that the S-layers can be important virulence factors. To help fight the respective diseases, these S-layers have been used for vaccine development[36–38]. Because S-layers have also been observed on numerous other human and animal pathogens, including *Chlamydia* spp., *Treponema* spp., *Campylobacter* spp., *Rickettsia* spp., *Wolinella* spp., *Bacteroides* spp., *Bordetella pertussis*, *Cardiobacterium hominis*, *Aeromonas* spp. and a great number of *Bacillus* spp. and *Clostridium* spp. (for a recent compilation see Refs 2,4,5), it can be assumed that they will also prove relevant as virulence factors for possible use in vaccines.

Box 1. Biotechnological and nanotechnological applications of S-layers

- S-layer ultrafiltration membranes (SUMs) with defined surface and molecular sieving properties.
- SUMs, S-layer microparticles (SMPs) or S-layer self-assembly products (SAPs) for the controlled immobilization of functional molecules:
  SUMs for covalent binding of enzymes for amperometric and optical bioanalytical sensors.
  SUMs with immobilized monoclonal antibodies for dipstick-style immunoassays.
  SMPs with immobilized Protein A for escort-particles in affinity cross-flow filtration for isolation and purification of antibodies.
  SMPs with immobilized antibodies for preparation of microparticles for ELISA.
  SAPs as carriers with intrinsic adjuvant property for weakly immunogenic antigens and haptens.
- S-layers as supporting structures for Langmuir–Blodgett films and liposomes with incorporated functional molecules:
  S-layer coated liposomes with surface-located, covalently bound target molecules.
  S-layer coated liposomes with immobilized antigens and haptens for vaccination.
- Genetic manipulation of S-layer proteins for production of self-assembling fusion proteins:
  Incorporation of functional domains as required for affinity matrices, vaccines, biosensors and diagnostics.
- Matrices for controlled biomineralization.
- Templates and nanonatural resists for semi-conductor technology.

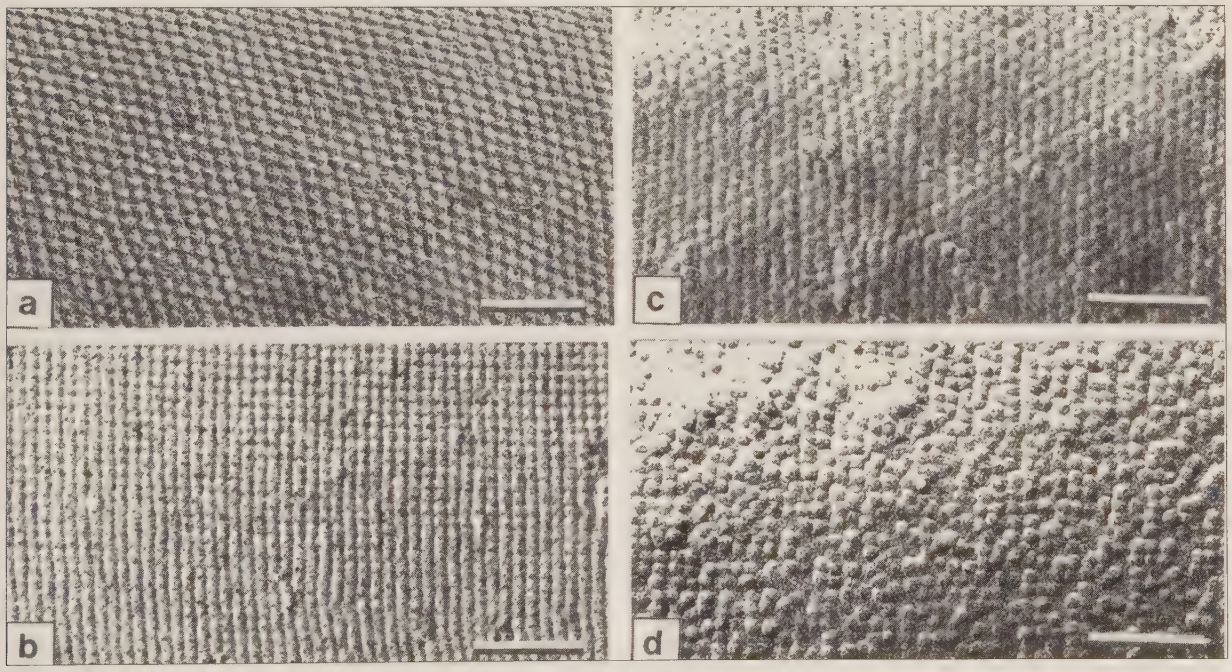

Figure 2
Freeze-etching preparations showing a hexagonal and a square S-layer lattice before (**a,b**) and after (**c,d**) immobilization of ferritin. The covalently bound ferritin reflected the hexagonal (**c**) and square (**d**) pattern of the respective underlying S-layer lattice. Scale bars, 100 nm.

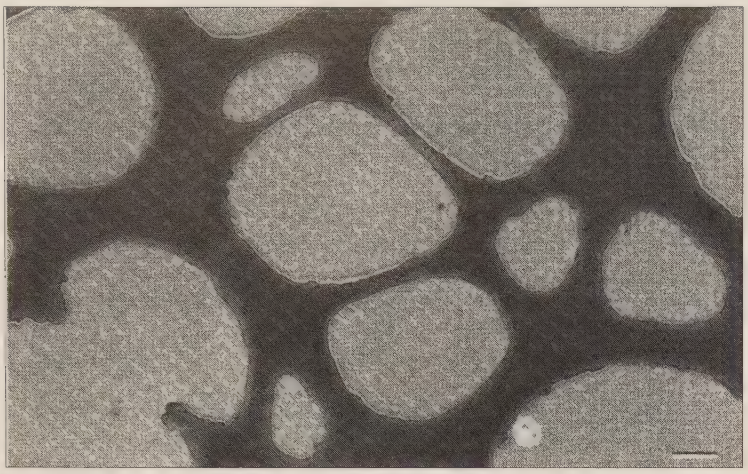

Figure 3
Electron micrograph of a negatively stained preparation of a porous support completely covered with the S-layer protein of *Bacillus sphaericus* CCM 2177 recrystallized onto a tetraetherlipid monolayer film. Scale bar, 500 nm.

Another line of vaccine development has been the use of SAPs as immobilization matrices for weakly immunogenic antigens and haptens[39–42]. Moreover, S-layer vaccines were shown to possess an intrinsic adjuvant property[39]. Both glycosylated and non-glycosylated S-layer lattices have been used as vaccine carriers. So far, immunogenic conjugates have been prepared using S-layers conjugated to tumor-associated oligosaccharides[39], to oligosaccharide fragments derived from *Streptococcus pneumoniae* capsular polysaccharides and to the recombinant major birch pollen allergen[39]. S-layer vaccines offer several advantages over conventional combinations of carrier proteins and adjuvant. Their injection by intradermal or intramuscular routes does not cause measureable trauma or side-effects. Furthermore, immune responses to the hapten S-layer conjugates have been observed following topical oral/nasal application. More recently, investigations have also focused on target indications in which immune effector mechanisms mediated by the Th1 helper cell subset are important[41,42].

Generally, S-layers as carriers could have a significant advantage in comparison with the widely used tetanus and diphtheria toxoids. Secondary and tertiary immunization could be performed using the same hapten(s) or antigens coupled to different immunologically non-crossreactive S-layers, thus preventing the tolerance problem frequently observed with toxoid carriers.

S-layers as supporting structures for functional lipid membranes

The potential of reconstituted (at macroscopic dimensions) biological membranes incorporating specific functional attributes (e.g. ion channels, carriers, pore-forming proteins, proton pumps, receptors) has already attracted much attention, in both basic and applied research. Because lipid membranes in their functional state are dynamic, fluid structures, long-term investigations of the structure, function and application potential are frequently hampered by their short life and low mechanical stability. One of the main reasons for the disintegration of unsupported lipid membranes is the transversal vibration of the constituent lipid molecules. The observation that the cell envelope of many archaea that grow under extreme environmental conditions (e.g. low pH, high salt concentrations or high temperatures) is composed of an S-layer and an associated cytoplasmic membrane led to the concept of stabilizing large-scale reconstituted functional lipid membranes with S-layer lattices. Large-scale monomolecular coherent S-layer lattices on lipid films spread at the air–water interface can be achieved by injecting isolated S-layer subunits into the subphase of Langmuir–Blodgett (LB) troughs[18,43,44]. These S-layer-supported LB-lipid films can cover holes of porous supports (e.g. apertures) up to several micrometres in diameter (Fig. 3) and still maintain their structural integrity during the course of subsequent handling when compared with unsupported lipid membranes[18]. This is especially true after crosslinking the S-layer proteins alone or with molecules from the lipid layer of the subphase (e.g. with glutaraldehyde). Lipid layers can be deposited on such 'semi-fluid membranes' (Ref. 43) by standard LB-techniques or by fusion of lipid vesicles. Functional molecules can be incorporated into S-layer-stabilized lipid layers using well-established procedures. This new technology has the potential to initiate a broad spectrum of developments in such areas as sensor technology, diagnostics, biotechnology and electronic or optical devices[18,29–31]. Presently, there are few alternative biomimetic approaches other than S-layer-supported lipid membranes available for exploiting the specific functional principles of biological lipid membranes at macroscopic dimensions.

It was recently demonstrated that S-layer subunits can also recrystallize on liposomes[45] composed of phospholipids and tetraetherlipids (Fig. 4). These S-layer-coated liposomes are closed biomimetic structures that resemble archaeal cell envelopes. The S-layer proteins, once recrystallized on liposomes, can be crosslinked and exploited as a matrix for the covalent attachment of molecules. Since liposomes have a broad application potential, particularly as carrier and/or drug delivery and drug targeting systems, this possibility for modulating the surface properties appears to be very important[29,45].

Conclusion and future directions

S-layers represent the simplest protein membranes developed during evolution. Their construction principle is based on a single constituent protein (or glycoprotein) subunit with the intrinsic ability to assemble into isoporous lattices possessing repetitive physicochemical properties down to the sub-nanometer scale. This unique feature makes S-layer structures that exist at the ultimate resolution limit for the molecular functionalization of surfaces and interfaces. A broad range of applications for S-layers has already emerged. Major

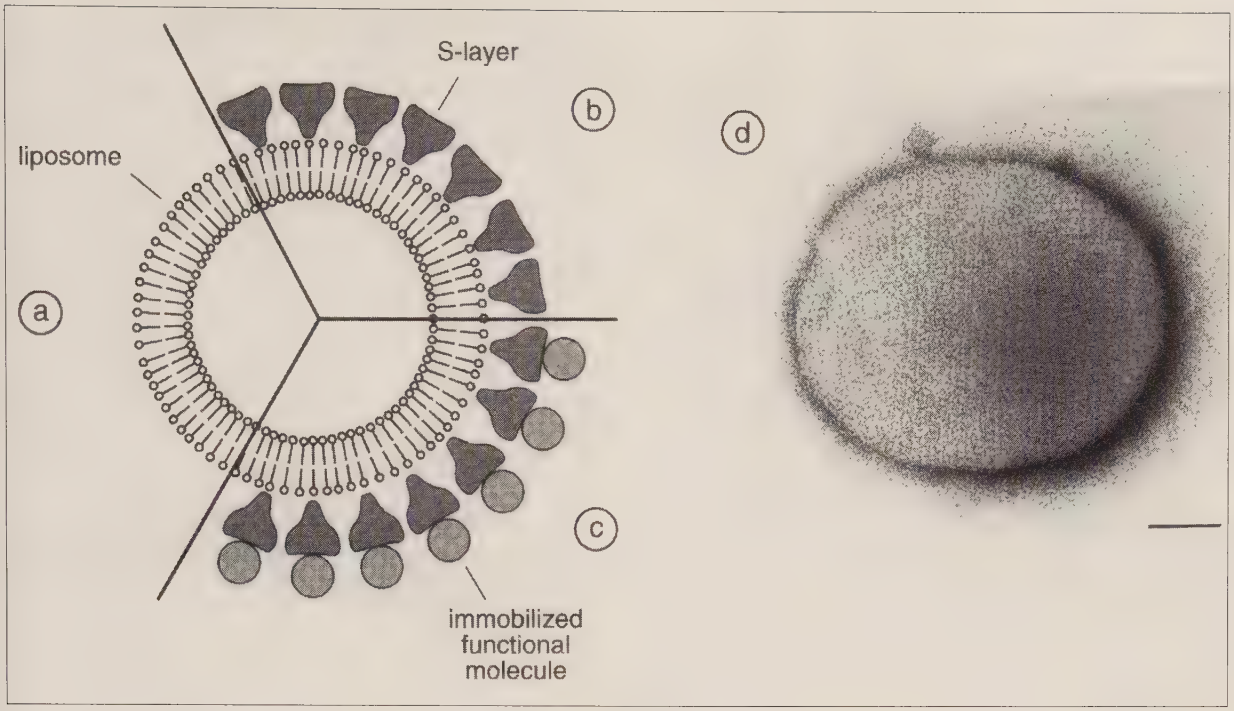

Figure 4

Schematic drawing of a liposome (**a**) coated with an S-layer lattice (**b**) that can be used as a matrix for the immobilization of functional macromolecules (**c**). (**d**) Negatively stained preparation of a liposome completely covered with the oblique S-layer lattice of *Bacillus coagulans* E38-66/V1. Scale bar, 100 nm.

areas for future development will be based on specific modifications of the unique molecular sieving properties of S-layer lattices and their potential as matrices for immobilizing dense monolayers of defined, oriented and aligned functional molecules. Improving technologies based on S-layer-stabilized artificial or biological lipid membranes should allow, for the first time, the exploitation of specific membrane functions on a macroscopic scale. Another line of development is directed towards the genetic manipulation of S-layer proteins. Incorporation of peptide stretches of well-known functional domains of other proteins should lead to completely new affinity and enzyme membranes, ion-selective binding matrices, microcarriers, biosensors, diagnostics, vaccines, or biocompatible surfaces or bioabsorbable systems for tissue regeneration.

Finally, the recrystallization of S-layer proteins on silicon wafers into coherent monolayers and their patterning by deep ultraviolet eximer laser radiation[46,47] should lead to new designs for functional surfaces, supported biomimetic membranes and matrices for controlled biomineralization[48].

Acknowledgements

Part of this work was supported by the Austrian Science Foundation Grant S72 and the Austrian Federal Ministry of Science, Transportation and the Arts.

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Effect of glycosylation on antibody function: implications for genetic engineering

Ann Wright and Sherie L. Morrison

Antibodies are able to both bind antigens and trigger the responses that eliminate them from circulation. All antibodies are glycosylated at conserved positions in their constant regions, and the presence of carbohydrate can be critical for antigen clearance functions such as complement activation. The structure of the attached carbohydrate can also affect antibody activity. Antibody glycosylation can be influenced by the cell in which it is produced, the conformation of the antibody and cell culture conditions. These variables should be considered in the design and production of antibodies with selected specificity and function.

Antibodies, or immunoglobulins (Igs), are serum proteins that play a central role in the humoral immune response, binding antigens and inactivating them or triggering the inflammatory response which results in their elimination. The antibody core structure is composed of two identical light chains and two identical heavy chains (Fig. 1). The light chains fold into two domains and the heavy chains into four or five[1]. The N-terminal domains of each chain constitute the variable (V) regions which recognize antigens, while the other domains make up the constant (C) regions. Antibodies form a Y-shaped structure consisting of two identical Fab 'arms', each composed of a light chain and the variable and first constant region of a heavy chain, and an Fc stem with

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PII: S0167-7799(96)10062-7

the remaining heavy chain constant domains (see Fig. 1). The Fab regions contain the antigen-binding sites, while the elimination of antigen is mediated by the Fc through the activation of host immune mechanisms such as complement and antibody-dependent cell-mediated cytotoxicity (ADCC). In humans, there are five classes of immunoglobulins: IgG, IgA, IgM, IgE and IgD, with four further subclasses of IgG and two of IgA. These nine isotypes differ with respect to biological activity, structure and relative abundance in serum and secretions.

All antibodies contain carbohydrate at conserved positions in the constant regions of the heavy chains, with each isotype possessing a distinct array of N-linked carbohydrate structures, which variably affects protein assembly, secretion or functional activity[1]. In addition, up to 30% of rabbit and human IgGs possess Fab-associated carbohydrate[2,3]. The structure of the attached N-linked carbohydrate varies considerably, depending upon the degree of processing[4,5], and can include high-mannose, multiply-branched as well as biantennary complex oligosaccharides (Fig. 2). IgA1 and IgD molecules also possess several O-linked sugars[6,7]. IgG, the most abundant antibody in serum, has a single N-linked biantennary structure at Asn297, which is buried between the C_H2 domains, forming extensive contacts with amino acid residues within C_H2 (Ref. 8; Fig. 3). The oligosaccharide is approximately as large as the C_H2 domains[9].

Antibodies have many structural and functional features that make them valuable reagents for research or therapy. They are capable of highly specific interactions with a wide variety of ligands, both in their Fab and Fc regions. Functional domains providing specific antigen-binding (Fabs or Fvs) or effector functions (Fcs) can be exchanged between molecules, expressed as separate, biologically active, fragments, or expressed as components of novel fusion proteins[10,11]. Such manipulations have been instrumental in elucidating the biological functions of the different Igs and have contributed to improvements in the design of genetically engineered antibodies.

Innovative gene expression systems have been developed to allow the production of intact antibodies or fragments in a variety of heterologous hosts such as lymphoid and non-lymphoid mammalian cells, bacteria, yeast, plants and insect cells[12]. The challenge is to define an expression system that produces antibodies with the desired functional properties. Because antibodies are glycoproteins, an additional concern is the contribution of the attached carbohydrate to their functional properties. Antibodies produced in prokaryotic cells lack carbohydrate[13]. Although all higher organisms are capable of glycosylation, both the structure of the attached oligosaccharide and the efficiency of glycosylation site utilization may vary.

This review will focus on IgG and will address the effects of the absence or alteration of carbohydrate structures on the functional properties of antibodies.

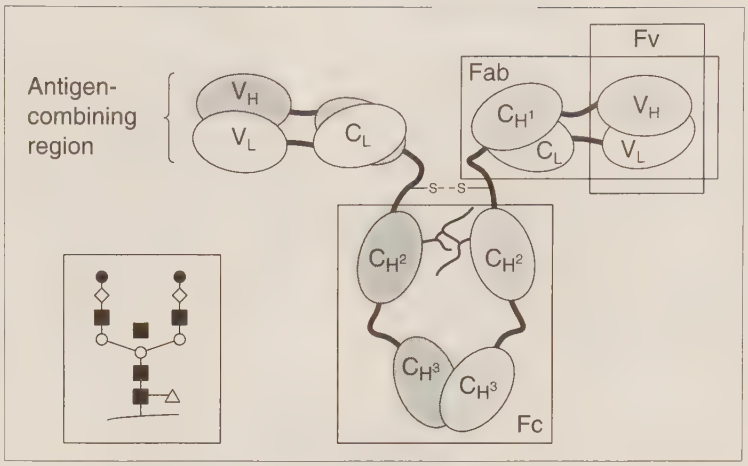

Figure 1

Schematic of a human IgG1 antibody molecule[1] composed of two identical light chains (each composed of two domains, labeled V_L and C_L) and two identical heavy chains (each with four domains, labeled V_H, C_H1, C_H2 and C_H3). IgG molecules can be cleaved enzymatically into several functional subunits: Fvs, composed of V_H and V_L; Fab fragments, which consist of the light chain and the V_H and C_H1 of the heavy chain; and the Fc, consisting of heavy chain constant domains. A flexible hinge separates Fabs from the Fc. Amino acid residues in the V_H and V_L domains form the antigen combining sites. Effector functions, such as complement activation and binding to cytotoxic cells, are mediated by the Fc region. The carbohydrates between the C_H2 domains are represented by forked structures. Inset: The composite, fully-processed carbohydrate structure attached to Asn297 in C_H2 (Ref. 55). Sugar residues include: *N*-acetylglucosamine (GlcNac; ■), mannose (Man; ○), fucose (Fuc; Δ), galactose (Gal; ◊) and sialic acid (SA; ●).The sugar residues are attached in invariant order to the core structure $GlcNac_2Man_3GlcNac$, but the actual numbers of the outer residues will vary.

The role of carbohydrate in IgG function

As mediators of the humoral immune response, antibodies trigger biological responses after binding to specific antigens by interacting through their Fc regions with both cellular and soluble effector systems. The binding site on IgG for C1q, the first component of the complement cascade, has been localized to the C_H2 domains (Fig. 3); antigen-mediated aggregation of IgG1, IgG2 or IgG3 initiates complement activation[14]. Binding of IgG to the high-affinity Fc receptors on monocytes can stimulate those cells to eliminate the antigen to which the Ig is bound. Other cells such as natural killer (NK) cells and neutrophils express low-affinity Fc receptors that produce a cytotoxic response when engaged by aggregated antibodies[15].

Removal of carbohydrate and its effect on function

Two general approaches have been used to determine the effect of total removal of oligosaccharide: tunicamycin treatment of the antibody-producing cells to inhibit the attachment of the oligosaccharide precursor to asparagine[16,17], or removal of Asn297 by mutation to Gln (Ref. 18). Although both manipulations profoundly depressed certain antibody functions, subtle differences were observed. In both cases interaction with antigen and *Staphylococcus* Protein A was unaffected. However, both C1q binding and monocyte-associated Fc receptor binding were undetectable in

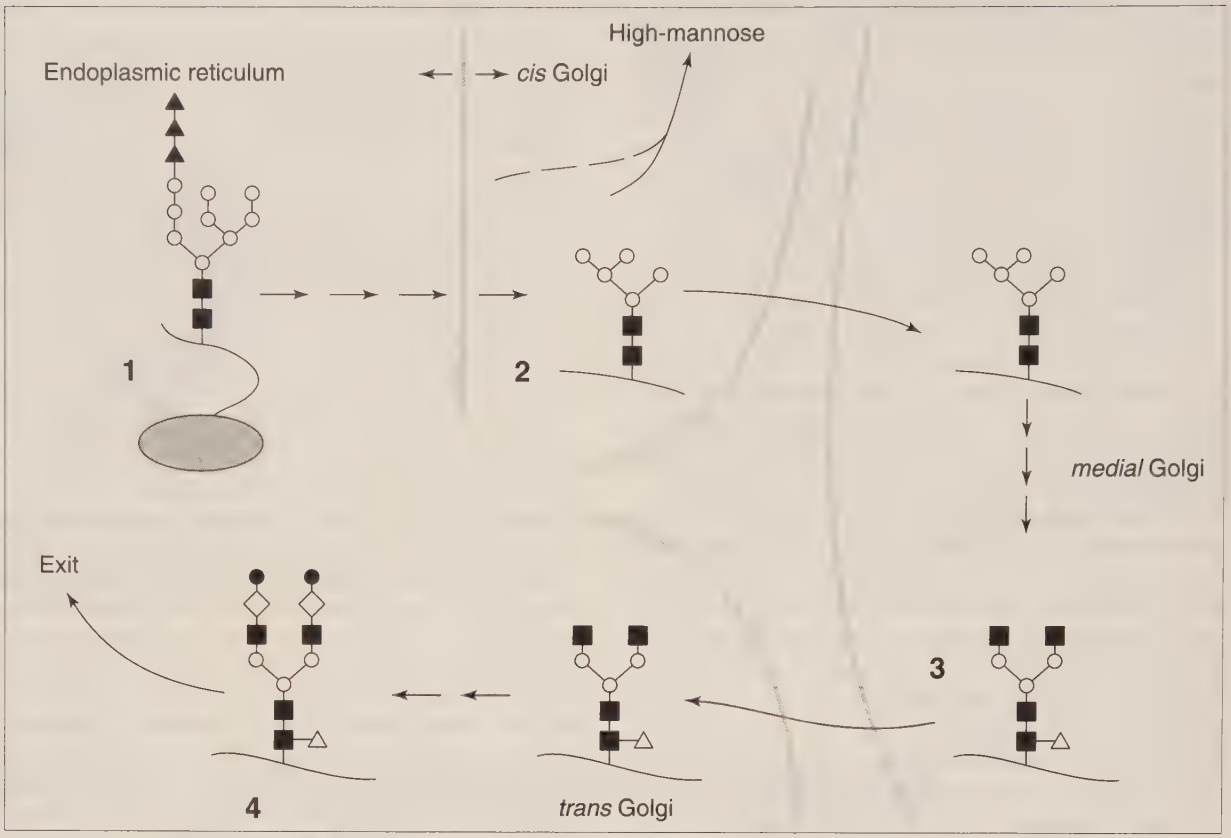

Figure 2

An abbreviated schematic representation of the processing pathway of oligosaccharide to complex biantennary form[51]. The newly synthesized species $Glu_3Man_9GlcNac_2$ (**1**) is transferred from dolichyldiphosphate to the Asn-X-Ser/Thr sequence in the peptide as it emerges from the ribosome. The arrows indicate sequential enzymatic reactions through which sugar residues are trimmed as the glycoprotein passes through the endoplasmic reticulum. After removal of the three terminal glucoses, the glycoprotein then moves to the *cis* Golgi, where it undergoes a series of steps through which mannose residues are trimmed by α-mannosidases. Processing can stop at this point, yielding glycoproteins with high-mannose sugars attached. Alternatively, processing can proceed to yield $Man_5GlcNac_2$ (**2**). This intermediate is the preferred substrate for *N*-acetylglucosaminyltransferase I, whose action, in the *medial* Golgi, is the committed step in complex oligosaccharide synthesis. The CHO glycosylation mutant Lec1 is deficient in this enzyme so the sugars produced by these cells bear this structure. In the *medial* (**3**) and *trans* (**4**) Golgi the oligosaccharide undergoes further processing steps in which mannose residues are trimmed and the sugar residues are sequentially added. The newly synthesized glycoprotein then exits the Golgi and is transported to the cell membrane or is secreted. Symbols: glucose (▲); mannose (○); *N*-acetylglucosamine (■); fucose (Δ); galactose (◊) and sialic acid (●).

the mutant aglycosylated antibodies, while these functions were reduced but not eliminated in the tunicamycin-treated antibodies. Tunicamycin-treated IgG3 showed loss of rosette formation through Fc receptors expressed on cultured tumor cells[17] and NK cells[19]. For Gln297 variants, the *in vivo* half-life of IgG1 was not affected by lack of carbohydrate, while that of IgG3 was shortened. Compared with wild type, aglycosylated IgG1 and IgG3 showed increased sensitivity to proteases and altered reactivity to a panel of monoclonal antibodies, suggesting that alterations in protein conformation had occurred.

More recently, when mouse IgG2a antibodies either treated with tunicamycin or deglycosylated by virtue of an Asn297→Ala mutation were compared directly for *in vivo* clearance and binding of the Fc receptor from neonatal rat (FcRn; Ref. 20), the genetically aglycosylated antibody was indistinguishable from wild type, whereas the tunicamycin-treated antibody was less stable, cleared more rapidly and was transported by FcRn less efficiently. It was concluded that tunicamycin treatment caused alterations in the protein structure in addition to removing the carbohydrate, suggesting that antibiotic treatment may introduce unintended variables.

Effect of altered carbohydrate structure on antibody function

Typically there is heterogeneous processing of the core oligosaccharide structures attached at a particular glycosylation site so that even monoclonal antibodies exist as multiple glycoforms[21–23]. While such heterogeneity is normal, certain structures appear to be associated with aberrant antibody function. The serum IgG of some patients with certain auto-immune diseases such as rheumatoid arthritis, Crohn's disease and osteoarthritis contains a higher than normal proportion of sugar structures

terminating in *N*-acetylglucosamine (Fig. 2). It has been suggested that such antibodies might be more susceptible to aggregation and formation of insoluble immune complexes[24]. To determine whether defined alterations in carbohydrate structure actually affect antibody properties, terminal residues have been selectively removed by treatment with specific glycosidases, or antibodies have been expressed in cells that attach sugars that are incompletely processed.

Exoglycosidase treatment of IgG antibodies

To obtain a population of serum IgG that was totally lacking galactose, Tsuchiya *et al.*[25] treated polyclonal IgG with neuraminidase and β-galactosidase. Compared with untreated samples, these antibodies revealed a significant reduction in C1q binding and Fc receptor binding, but were unaffected in their capacity for *Staphylococcus* Protein A and rheumatoid factor binding. Desialation of the CDw52-specific human monoclonal antibody Campath-1H produced no effect on any function tested[26]. Removal of galactose was found to reduce complement-mediated lysis of antigen-bearing target cells but did not affect ADCC. Although these experimental systems are not completely equivalent they suggest that degalactosylation of IgG1 will have a deleterious effect on complement activation but that the reduction in Fc-receptor-mediated activity varies depending on the subclass and assay.

Using human IgG that was enzymatically degalactosylated, Malhotra *et al.*[27] reported that the terminal *N*-acetylglucosamine residues thus exposed were accessible and could interact with mannose-binding protein (MBP), a serum protein with a structure similar to C1q. The deposition of C4b, an intermediate in the complement cascade, was observed when immobilized Fc fragments produced from these antibodies were incubated with MBP and serum. It was suggested that this unconventional capability to activate complement could contribute to the chronic inflammation associated with rheumatoid arthritis.

Treatment of cells with the carbohydrate-processing inhibitor swainsonine (Sw) results in the synthesis of 'hybrid' carbohydrate structures that possess one fully processed arm and one containing mannose exclusively. When hybridoma cells producing a sheep red blood cell (SRBC)-specific monoclonal antibody of the murine IgG2a subclass were treated with Sw (Ref. 28), the capacity of these antibodies to be secreted, bind antigen, mediate ADCC of SRBCs and perform complement-mediated hemolysis of target cells was unaffected. However, the new carbohydrate structure was accessible to lectin binding, whereas the biantennary structure is normally buried between the C_H2 domains[29]. Therefore, in this system, possession of even one fully processed arm of the sugar structure sufficed for retention of C_H2-associated function, although the aberrant sugar may introduce variations in conformation.

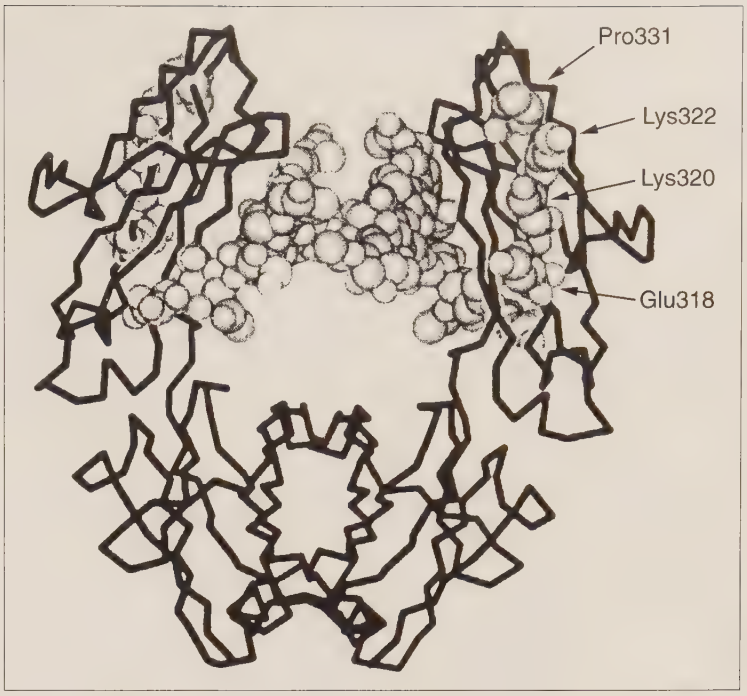

Figure 3

Crystal structure of the Fc portion of a human IgG1 molecule[52] depicted using the program *MacImdad*. The carbohydrate chains attached to Asn297 in each chain are shown in space-filling format, extending between the C_H2 domains. The residue Pro331, which is critical for high-affinity Fc receptor interaction and for complement activation[53], is located on a loop near the lower hinge region and is indicated at the top of the drawing. Also labeled are the charged residues Glu318, Lys320 and Lys322, which constitute a motif that is thought to play a role in Fc binding to C1q, the initial step in the classical complement activation pathway[54]. The lower hinge region is not resolved in this structure. For simplicity the amino acids of only one chain are labeled.

Rothman *et al.*[30] tested the capacity for ADCC of monoclonal murine IgG antibodies that were purified from hybridomas grown in the presence of glycosidase inhibitors that acted at different steps in the oligosaccharide-processing pathway. These inhibitors included Sw (see above) and castanospermine (Cs), which inhibits the removal of glucose residues from the oligosaccharide newly attached to the peptide (Fig. 2; compare Structure 1 to Structure 4). Compared with wild-type antibodies, those treated with Cs showed enhanced ADCC mediated by NK cells but not by other types of effector cells such as monocytes. By contrast, Sw-treated antibodies failed to induce enhanced NK-cell-mediated ADCC. Through lectin-binding analysis the oligosaccharides on Sw-treated and wild-type IgGs were shown to contain fucose, which was lacking on the Cs-treated antibodies. It was suggested that recognition by IgG Fc of the type of Fc receptor present on NK cells, leading to enhanced ADCC, was glycosylation dependent, requiring the absence of fucose. However, both Sw-treated and wild-type oligosaccharides contain at least one complex 'arm', which would produce an overall conformation, as well as several sugar residues, that differs from the oligosaccharide produced by Cs treatment.

Studies with antibodies produced in glycosylation mutants

Chinese hamster ovary (CHO) cells are commonly used to produce many recombinant proteins, including immunoglobulins. These cells attach sugar structures that resemble those found on human cells[31,32]. Immunoglobulins have been produced in CHO cells that have defined defects at distinct steps in the oligosaccharide biosynthesis pathway[33], resulting in the accumulation of intermediate structures with reduced overall carbohydrate heterogeneity. The CHO glycosylation mutants are useful for analyzing specific alterations in carbohydrate structure in normal proteins, without resorting to the use of glycosidase treatment that might affect the protein backbone.

In initial studies, IgG1 antibodies were produced in Lec1 cells, which lack *N*-acetylglucosaminyltransferase I activity, leading to the accumulation of high-mannose intermediates not normally found on IgG (Fig. 2; Ref. 34). When compared with IgG produced in wild-type myeloma or CHO cells, the Lec1-produced antibodies retained antigen specificity but were incapable of complement-mediated hemolysis, were greatly deficient in C1q binding and complement consumption, and showed reduced but significant affinity for the high-affinity Fc receptors. The *in vivo* half-life of the Lec1-produced antibody was shorter than that of the normally glycosylated antibodies, although clearance could be temporarily blocked by the co-injection of yeast-derived mannan. More recently, antibodies have been expressed in CHO glycosylation mutants deficient in the attachment of either sialic acid or galactose, resulting in glycoforms that are normally seen on serum IgG.

Glycosylation profoundly affects the effector functions of IgG antibodies and the glycosylation status of IgG can be altered either enzymatically or by manipulating the glycosylation machinery of cells. Such studies will help determine whether particular carbohydrate structures definitively affect antibody function and may lead to the optimization of expression systems or growth conditions for the production of such glycoproteins.

Factors affecting antibody glycosylation

Expression systems and glycosylation

Comparative studies of IgGs obtained from different species have reported species-specific differences in IgG-associated oligosaccharide structures. For example, whereas a significant proportion of human IgG antibodies attach an *N*-acetylglucosamine (GlcNAc) residue that bisects the biantennary sugar[35], antibodies produced in murine cells lack that structure[21,22]. Furthermore, CHO cells attach sialic acid through ($\alpha2\rightarrow3$) linkages exclusively, whereas human and murine cells can attach a mixture of ($\alpha2\rightarrow3$) and ($\alpha2\rightarrow6$)-linked sialic acid residues. While human Igs preferentially attach galactose residues to the Man($\alpha1\rightarrow6$) branch of the sugar, galactosylation of the Man($\alpha1\rightarrow3$) branch predominates in bovine Igs (Ref. 36).

In a few instances, antibodies produced in murine cells contain terminal ($\alpha1\rightarrow3$)galactose[23,37], which has been identified in both the Fc and constant regions of antibodies. This carbohydrate residue is widely distributed among non-primate mammals and in man is recognized by up to 1% of circulating Ig (Ref. 38). Thus, antibodies bearing that residue would probably be subject to rapid immune clearance, greatly compromising their effectiveness as *in vivo* therapeutic agents[39].

While subtle differences in IgG glycosylation are observed among mammalian cells, they all attach a biantennary structure with a common core at Asn297. However, production of immunoglobulins in non-mammalian expression systems can introduce considerable variation in carbohydrate structure. Insect cells produce truncated sugars and also are reported to attach xylose[40]. Yeast attach high-mannose sugars of up to 200 residues[41]. Antibodies thus glycosylated retained ADCC but lost the ability to activate complement[42].

Plants have been proposed as an economical method for the large-scale production of recombinant antibodies[43]. Plant cells elaborate complex carbohydrates through a biosynthetic pathway similar to that of mammalian cells, but can attach a xylose residue – which is not found in mammals – linked to the β-linked mannose of the glycan core. Sialic acid is absent in plants[44]. To date, the question of whether these novel structures affect antibody function has not been adequately addressed. Antibodies produced in plants reportedly bind antigen effectively; however, antigen binding is generally not influenced by Fc-associated glycosylation. It is not clear whether Fc-associated functions have actually been tested in the plant-produced antibodies.

Effect of protein conformation on glycosylation

The degree of processing of a carbohydrate at a particular attachment site on a protein depends on the accessibility of that site to the relevant glycosyltransferases. Microheterogeneity of Fc-associated carbohydrate structures exists with polyclonal human serum IgG containing approximately 30 different biantennary structures[35]. Studies of glycosylation of different IgG subclasses have reported that the distribution of galactose on IgG2 appears to differ from the other subclasses[45,46]. It was suggested that differences in protein conformation might affect the accessibility of the arms of the biantennary structure to galactosyltransferases; however, only a small number of proteins has been analyzed and clonal variation cannot be discounted. As a rule, novel sugar structures have not been observed, although in one comparative study of IgG myeloma proteins[46] small amounts of C_H2-associated high-mannose sugars were found to be attached to IgG2 and IgG3 paraproteins.

The adjacent immunoglobulin structure itself can affect glycosylation. Comparison of glycosylated Bence-Jones (free light-chain) proteins with Fab fragments produced from IgGs containing a glycosylation

site in the light chain showed the Bence-Jones proteins to be much more uniformly glycosylated, suggesting that the glycosylation site in the free light chain is more accessible to glycosyltransferases[45]. Indeed, Fab-associated glycosylation sites tend to be more fully processed than Fc-associated sites within the same molecule[35,45], suggesting a difference in accessibility. Thus the direct expression of antibody fragments may lead to differences in glycosylation that might have functional or physiological significance.

The carbohydrate associated with C_H2 in IgG forms extensive contacts with amino acids within that region. In a recent study these amino acid residues were altered by site-directed mutagenesis. It was found that mutation of a contact residue for a core *N*-acetylglucosamine, but not for galactose, resulted in a loss of recognition of that Fc region by Fc receptors[8]. Moreover, mutations of several contact residues resulted in increased sialation and galactosylation of the oligosaccharide chains[47].

Culture conditions and their effect on glycosylation

A pressing concern of commercial antibody production is the delivery of large quantities with high and consistent quality while minimizing cost. Cell culture methods for IgG production have been developed and compared. Both human[48] and chimeric mouse–human IgG[23] were more heavily galactosylated when grown in still culture than in hollow fiber reactors. The highly-galactosylated human IgGs showed enhanced ADCC involving both low- and high-affinity Fc receptors. However, it is not clear whether *in vivo* clearance or any other biological functions were affected. Cell culture conditions such as pH, concentration of NH_4^+ and hormonal supplements have been shown to affect N-linked glycosylation in several systems[49,50].

Conclusion: manipulation of glycosylation

Glycosylation is an inherently complex process whose purpose appears to be to introduce diversity into cells and proteins. Although it is clear that correct glycosylation is critical for effector activity of antibodies, the experimental systems used are not entirely comparable so the results may be incomplete and are not always entirely consistent. Nevertheless, some findings suggest that some alterations in glycosylation may allow the 'uncoupling' of certain functions (e.g. antibodies that can bind Fc receptors but cannot activate complement). These observations may eventually add a new dimension to the deliberate design of antibodies with a selected specificity and function. Moreover, the ability to produce quantities of antibodies with specific alterations in carbohydrate structure may help resolve the role of carbohydrate in antibody function.

Acknowledgements

Research in this laboratory was supported by grants CA-16858, AI-39187 and AI-29470 from the National Institutes of Health and ACS-IM-77313 from the Cancer Society.

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'Interfacial activation' of lipases: facts and artifacts

Robert Verger

An important aspect of lipolytic enzymes is the unique physicochemical character of the reactions they catalyse at lipid–water interfaces, involving interfacial adsorption and subsequent catalysis *sensu stricto*. Lipases are now used as catalysts in aqueous as well as low-water media and accept various molecules as substrates. They were previously defined in kinetic terms, based on 'interfacial activation'. This phenomenon was not found among esterases. Recently determined 3D structures of some, but not all, lipases show a 'lid' controlling access to the active site. Thus, the presence of a lid, and 'interfacial activation', are unsuitable criteria for classifying specific esterases. Consequently, lipases can be pragmatically redefined as carboxyl-esterases acting on long-chain acylglycerols: they are simply fat-splitting 'ferments'.

Three of the four main classes of biological substances, carbohydrates, proteins and nucleic acids, have been clearly defined on the basis of their structural features, whereas the property that is common to all lipids, the fourth main class, is a physicochemical one. Lipids are in fact a group of structurally heterogeneous molecules that are all insoluble in water but soluble in apolar and slightly polar solvents such as ether, chloroform and benzene. Lipids have been classified by Small[1] according to their behaviour in the presence of water.

Conventional enzyme kinetics is of little relevance under heterogeneous conditions. To determine kinetic parameters, *in vitro* experiments have traditionally been carried out using synthetic short- and medium-chain esters as putative lipase substrates, such as methyl butyrate, triacetin (TC2), tripropionin (TC3) and tributyrin (TC4). In aqueous media, these esters can exist either as an isotropic solution in the form of monomers, micelles and adsorbed monomolecular films, or as a turbid emulsion, which appears at higher substrate concentrations. These various physicochemical forms of ester molecules are schematically depicted in Fig. 1 and they usually coexist as a complex equilibrium between potential lipase 'monomeric substrate' (S_1) or 'super-substrates' (S_2, S_3 and S_4).

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Definition of lipases and 'interfacial activation'

What exactly is a lipase? Is it enough to define it as a carboxyl-esterase that specifically hydrolyses acylglycerols? In 1958, Sarda and Desnuelle[2] defined lipases in kinetic terms, based on the phenomenon of 'interfacial activation'. This property was not to be found, for example, among enzymes that have been classified as esterases, i.e. those acting only on

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PII: S0167-7799(96)10064-0

carboxylic ester molecules that are soluble in water. The phenomenon of interfacial activation was, in fact, first observed in 1936 by Holwerda *et al.*[3] and then in 1945 by Schønheyder and Volqvartz[4]. It amounts to the fact that the activity of lipases is enhanced on insoluble substrates (such as emulsions) compared with the same substrates in true monomeric solutions. It therefore emerged from these studies that lipases might be a special category of esterases that are highly efficient at hydrolysing molecules having a carboxylic ester group and that are aggregated in water. This property was used for a long time to distinguish between lipases and esterases.

In 1990, however, the first two lipase 3D structures were elucidated. In human pancreatic lipase (HPL) as well as in *Rhizomucor miehei* lipase a surface loop, i.e. the lid domain, covers the active site, which is inaccessible to solvent. It was postulated that this loop had to undergo a conformational change in the presence of a lipid–water interface and this might explain the phenomenon of interfacial activation described by Sarda and Desnuelle[2] in the particular case of porcine pancreatic lipase (PPL). Subsequently, both lipases were co-crystallized in the presence of either inhibitors or mixed micelles and the hypothetical movement of the lid domain was then clearly demonstrated.

The original lipase definition of Sarda and Desnuelle[2] has been transformed progressively to cover an idealized concept, far removed from experimental facts and artifacts. A conceptual shift recently occurred, however, as the result of which interfacial activation has been univocally taken to mean a hypothetical conformational change occurring as the result of interfacial adsorption, as was originally postulated by Desnuelle *et al.*[5] Worse still, interfacial activation has sometimes been wrongly taken to refer to the increase in catalytic activity of an enzyme on a triacylglycerol substrate occurring either upon mechanical emulsification or in the presence of a tensioactive agent. In such cases, the increase in reaction rate is simply due to an increase in interfacial activity.

Recent lipase research has nevertheless shown how careful one has to be about extrapolating any kinetic and/or structural characteristics observed to lipases in general. The catalytic activity of many lipolytic enzymes has been measured using carboxylic esters that are partly soluble in water, and many differences have been found between the resulting profiles.

The same substrate (TC2), which is partly soluble in water, and the same purified lipase from porcine pancreas gave very different rate profiles depending upon the presence of sodium chloride[6] (Fig. 2b) or gum arabic (GA)[2] (Fig. 2a) in the assay medium. These differences can be understood by taking into account the fact that media of high ionic strength will generally reduce the value of the critical micelle concentration, with a corresponding increase in the size of micelles. Consequently, in the experiments carried out by Entressangles and Desnuelle[6] at 0.1 M NaCl, the presence of isotropic aggregates (micelles?) below the solubility limit could explain the relatively high catalytic activity found in contrast to Sarda and Desnuelle[2], who performed their kinetic experiments at a lower ionic strength and in the presence of GA. Furthermore, Sarda and Desnuelle[2] reported that purified fractions of PPL were relatively less active than crude fractions (which may have been contaminated by pancreatic esterases) when tested on an isotropic solution of TC2 (Fig. 2a). Thus these authors radically proposed that lipases, by contrast with esterases, are enzymes completely unable to act on a true monomeric solution of ester.

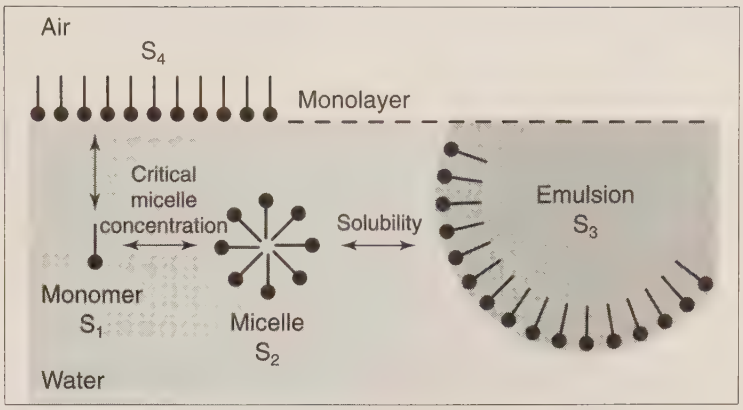

Figure 1
Schematic illustration of the various physicochemical states of ester molecules in aqueous media. Monomer (S_1), micelle (S_2), emulsion (S_3) and adsorbed monolayer (S_4), coexist in equilibrium and represent potential lipase 'monomeric substrate' (S_1) or 'super-substrates' (S_2, S_3 and S_4). The lighter area represents the isotropic solution and the darker area represents the turbid emulsion.

'Interfacial activation' of lipases: facts and artifacts

Extreme caution is necessary when both performing and interpreting kinetic measurements with lipids. First, it is essential to check that the initial lipase velocity is proportional to the amount of enzyme used, both below and above the solubility limit. Substrate depletion in the 'monomeric' range of substrate concentration is sometimes a major experimental limitation. Second, it is also essential to check that the same lipase active site, and not other, unspecific sites, is responsible for the measured catalytic activity on monomeric substrates. Control experiments with non-enzymatic proteins or inhibited lipase should be performed. Third, because the medium is heterogeneous, adding

Glossary

TC2 – Triacetin (triethanoylglycerol).
TC3 – Tripropionin (tripropanoylglycerol).
TC4 – Tributyrin (tributanoylglycerol).
GA – Gum arabic.
PPL – Porcine pancreatic lipase.
r-GPL-RP2 – Recombinant guinea-pig pancreatic lipase related protein 2.
Chimera – N-GPL-RP2/C-HPL chimera.
HPL – Human pancreatic lipase.

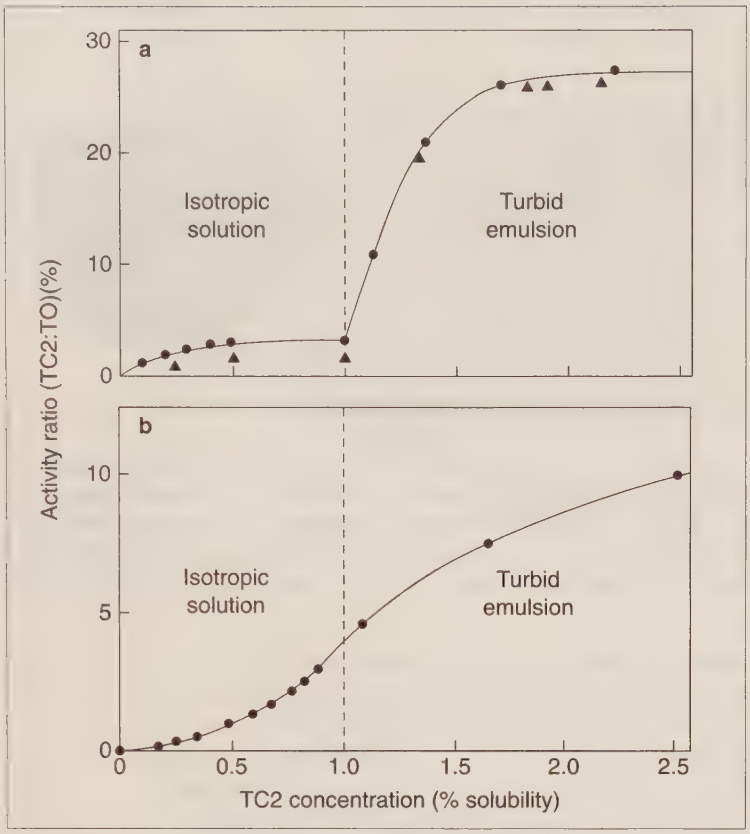

Figure 2

(**a**) Hydrolysis rate of triacetin (TC2), emulsified in gum arabic, as a function of substrate concentration. (From Ref. 2). The ordinates are expressed as a percentage of the maximal rate of the triolein (TO). Symbols: crude lipase preparation (specific activity of 400 μmol min^{-1} mg^{-1}) (●); purified lipase preparation (specific activity of 4230 μmol min^{-1} mg^{-1}) (▲). (**b**) Hydrolysis rate of TC2 as a function of substrate concentration. (From Ref. 6.) The ordinates are expressed as a percentage of the maximal rate observed on TO. Initial hydrolysis rates were measured titrimetrically in 0.1 M NaCl with a radiometer pH-stat at 25°C and pH 7.0 in a thermostatted vessel. The total concentration of the substrate (solution + emulsion) is expressed in multiples of the saturation. The vertical dotted line indicates the saturation point.

any amphiphilic compound to the system is liable to modify both quantitatively and qualitatively the physicochemical properties of the interface. For example, the presence of a phospholipid substrate in the micellar state will lead to the aggregation of pancreatic phospholipase A_2 into multimolecular lipoprotein complexes[7]. On the other hand, some lipases, such as gastric lipases, rapidly become denatured at an interface with a pure TC4 emulsion. Consequently, it is impossible to assess experimentally what interfacial activation may have occurred with substrates of this kind. In addition, some esters that are partly soluble in water sometimes form monomolecular adsorption films on the surface of the air bubbles produced by stirring the reaction mixture. This artifact is responsible for the great disparity found among initial velocity measurements when mechanical stirring methods of different efficiencies are used. It has been established that the lipid–water 'interfacial quality', in terms of interfacial tension, is one of the most decisive parameters to be considered when working with lipolytic enzymes[8]. This, unfortunately, means that valid comparisons can be made only between data obtained under strictly identical conditions, preferably at the same laboratory. This is why we decided to re-evaluate critically the phenomenon of interfacial activation using as substrates synthetic short-chain triacylglycerols, such as TC2, TC3 and TC4. We also measured the aqueous solubility of these short-chain triacylglycerols by two independent and complementary methods (Fig. 3).

The maximal decrease in the superficial tension indicates the point at which a closely packed adsorbed monolayer is formed at the air–water interface, in equilibrium with the monomeric solution. This point also corresponds to the appearance of an isotropic micellar solution[1]. By contrast, the turbidimetric method reveals the appearance of

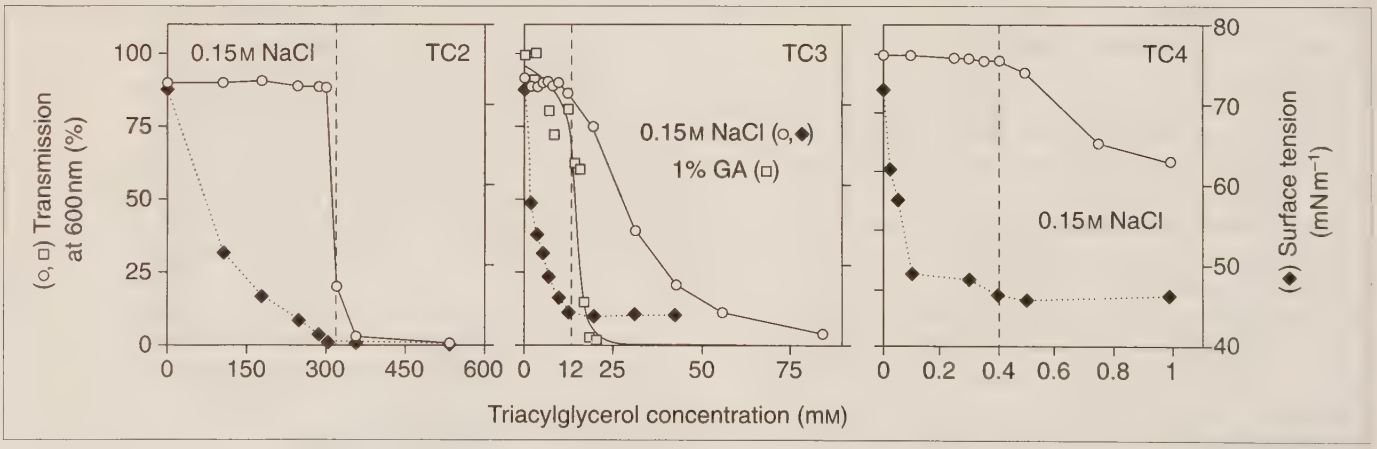

Figure 3

Aqueous solubility of short-chain triacylglycerols used as lipase substrates. For measurement of the decrease in the superficial tension[9,10] (◆), a Beckman LM 600 electromicrobalance was used with a platinum plate attached to the beam of the balance; the superficial tension was monitored and the equilibrium value was obtained after approximately 1 min. For turbidity measurements [○ in 0.15 M NaCl and □ in 1% gum arabic (GA)], the triacylglycerol solutions were poured into a 3 ml quartz cuvette placed in a thermostatted jacket (37°C), and the optical density was measured at 600 nm using a Kontron Uvikon 810 spectrophotometer. For tributyrin (TC4), the solutions used were the same as those in the superficial tension measurements. Each 250 ml volumetric flask was hand shaken before each turbidity measurement. TC2, triacetin; TC3, tripropionin.

large droplets, indicating that the solubility limit is reached.

Both the superficial tension and the turbidity measurements gave comparable values of the solubility of TC2 (318 mM) and TC3 (12 mM) that are very close to those reported previously by Entressangles and Desnuelle[6]. However, in the case of TC4 we found a value of the solubility limit close to 0.410 mM (Fig. 3). We then decided to repeat the experiments carried out originally by Sarda and Desnuelle[2], in 1958, using TC2 as substrate. However, in addition to PPL we used two other recombinant pancreatic lipases: the recombinant guinea-pig pancreatic lipase related protein 2 (r-GPL-RP2) and a chimeric r-GPL-RP2 mutant in which the C-terminal domain was substituted by the HPL C-terminal domain, as described recently by Carrière *et al.*[11]. The kinetic data are illustrated in Fig. 4.

As expected, PPL in the presence of its cofactor colipase displays a clear interfacial activation in the absence or presence of GA, in sharp contrast to r-GPL-RP2 and its chimeric mutant. These latter enzymes possess a large deletion within the lid domain, giving a free access to the lipase active site. The lack of interfacial activation in r-GPL-RP2 is probably a consequence of a maximal enzymatic activity being displayed on monomeric substrates[12]. In fact interfacial activation of classical lipases may be viewed as a depressed action on monomeric esters rather than an increased interfacial activity on aggregated substrates. In that sense, r-GPL-RP2 is a true lipase, distinguished by the absence of interfacial activation. The phenomenon of interfacial activation in HPL is probably linked to the ability of the lid domain to undergo a conformational change from an open to a closed conformation in the presence of a lipid–water interface. These conformations are probably stabilized by structural elements that are only present in the homologous protein core of HPL (Refs 11 and 13).

As reported previously by Entressangles and Desnuelle[6] (Fig. 2b), we found that the maximal

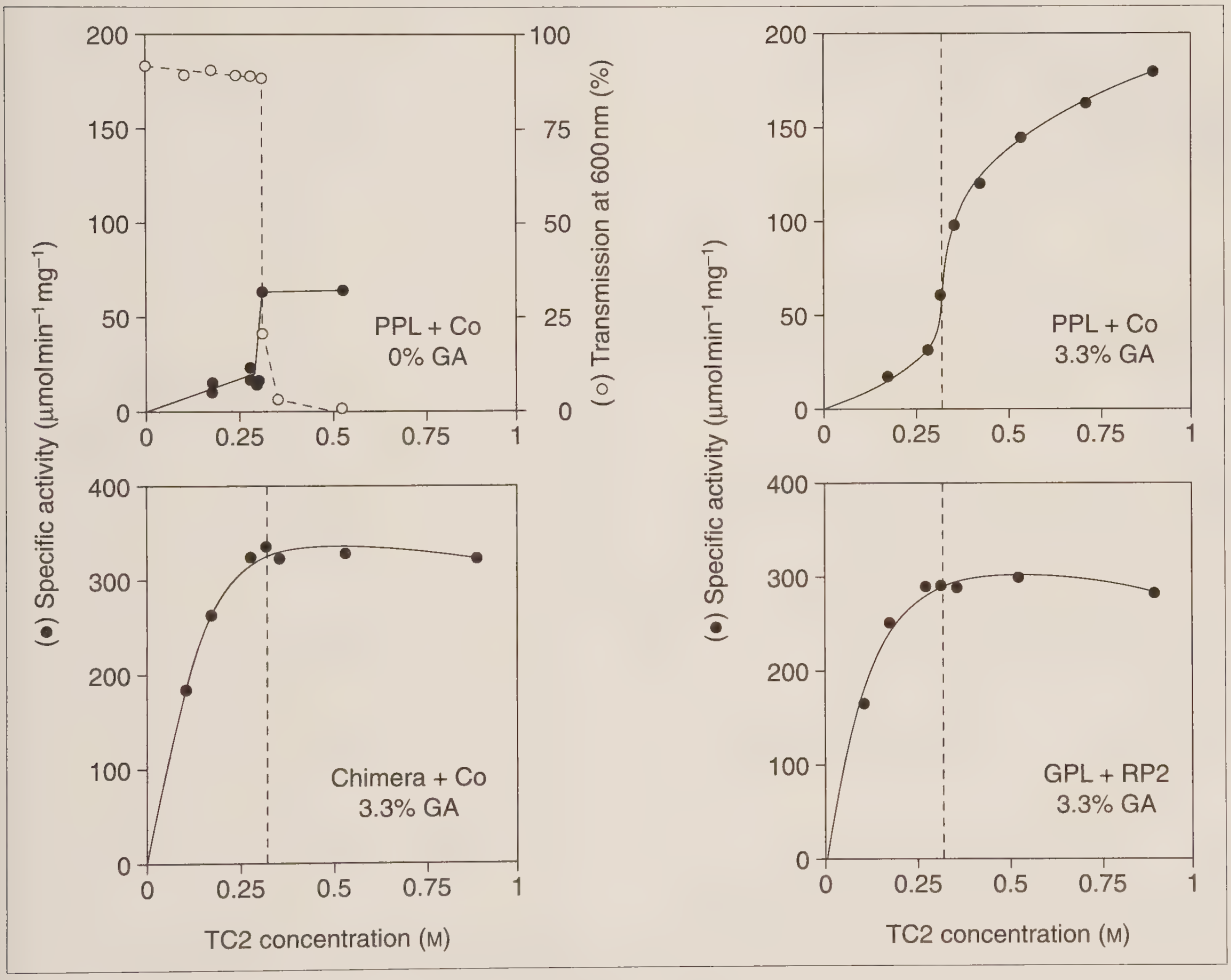

Figure 4

Triacetin (TC2) as substrate for measuring the interfacial activation of porcine pancreatic lipase (PPL), recombinant guinea pig pancreatic lipase (r-GPL-RP2) and the chimera. TC2 solutions were systematically prepared by mixing (3 × 30 s in a Waring blender) a given amount of TC2 in 15 ml of 10% gum arabic (GA). Before each assay, 5 ml of the above solutions were added to 10 ml of purified water (Millipore super Q system) in a thermostatted (25°C) pH-stat vessel. The pH-stat (TTT 80 radiometer) was equipped with a 250 μl syringe containing 0.1 M NaOH. The lipase activity was measured potentiometrically at pH 7.0, because at pH 8.0 the spontaneous hydrolysis of TC2 reaches relatively high levels. The PPL as well as the chimera samples used were saturated with a fivefold molar excess of colipase (Co). Symbols: specific activity (●); turbidity (○).

enzymatic activity expressed by PPL on TC2 was around 10% of the rate measured on a triolein emulsion. To find an acceptable compromise between lipase turnover rates and the water solubility of substrates, we used TC3.

From the results presented in Fig. 5, it is clear that TC3, emulsified in GA, is by far the best system to show the interfacial activation phenomenon in the case of PPL. It is also clear that in the absence of GA (i.e. in pure water) (see Fig. 5a), the lipase activity declined with time. As a result of the non-linear kinetics observed, which were possibly owing to an irreversible interfacial denaturation, the measurements of initial velocities are inaccurate and irreproducible. The presence of 0.15 M NaCl improves the assay conditions, allowing the interfacial activation phenomenon to be seen (Fig. 5b). It is worth noting from Fig. 5 that increasing concentrations of GA gradually shift the take-off point of lipase activity, up to TC3 concentrations above its solubility limit (12 mM). We have no plausible explanation for such an odd observation. However, it is worth noting that GA is basically a natural and complex polysaccharide (from the acacia tree) with poorly defined physicochemical properties and has been used since antiquity in pharmaceutical preparations. As a conclusion, interfacial activation can best be shown in a reproducible manner using TC3 as substrate, if one can avoid the many possible experimental artifacts discussed above.

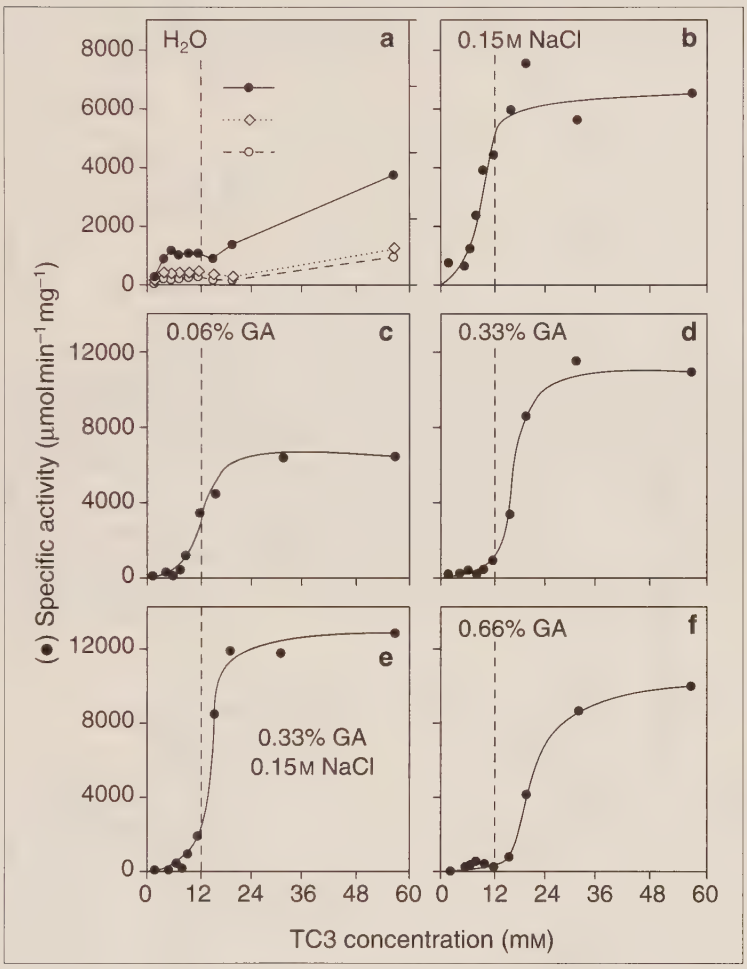

Figure 5

Tripropionin (TC3) as substrate for measuring the interfacial activation of porcine pancreatic lipase (PPL). The TC3 solutions were systematically prepared by mixing (3 × 30 s in a Waring blender) a given amount of TC3 in 15 ml of either pure water or 0.15 M NaCl or gum arabic (GA). The PPL samples used were saturated with a five-fold molar excess of colipase and (**a**) 7.6, (**b,d,e**) 1.52, (**c**) 0.86 or (**f**) 2.17 μg of PPL were injected. In experiments performed in pure water (**a**), non-linear kinetics were recorded. The rate of hydrolysis decreased with time and the instantaneous velocities were measured at 0 (●), 5 (◇) and 10 (○) min after lipase injection. The solubility limit of TC3 (12 mM) is indicated by vertical dotted lines. We checked that bovine serum albumin (1%, final concentration) had no detectable catalytic activity on a TC3 solution (7.7 mM) or on a TC3 emulsion (15.33 mM).

Are any structural elements responsible for 'interfacial activation'?

The first 3D structures of lipases to be elucidated[14,15] suggested that the interfacial activation phenomenon might be due to the presence of an amphiphilic peptidic loop covering the active site of the enzyme in solution, just like a lid. When contact occurs with a lipid–water interface, a conformational rearrangement might open this lid, making the active site accessible. It is worth noting, however, that the hydrolysis of a substrate having the form of a truly monomeric solution might also require the lid to be open without interfacial activation being involved. Interfacial activation seems connected with the respective lifetimes of the open and closed forms of lipases (see Fig. 6).

The European BRIDGE-T-Lipase project (1990–1994) produced some new 3D structures as well as numerous biochemical and kinetic data which provided new insights into lipases at the molecular and atomic levels[18]. It emerged from these studies that not all lipases subscribe to the rule of interfacial activation. The main exceptions to this rule, noted so far, are the lipases from *Pseudomonas glumae* (A. Deveer, PhD Thesis, University of Utrecht, 1992; Ref. 19), *P. aeruginosa*[20,21] and *Candida antarctica* B (Ref. 22), all of which nevertheless have an amphiphilic lid covering the active site.

Some new pancreatic lipases have been recently identified[23]. Comparisons between their primary amino acid sequences have shown that they have a fairly high degree of sequence identity but they can, nevertheless, be divided into three subgroups: (a) 'classical' pancreatic lipases; (b) type 1 (RP1) pancreatic lipases; and (c) type 2 (RP2) pancreatic lipases. Although the kinetic properties of the 'classical' pancreatic lipases, particularly as regards interfacial activation, have been well documented, two members of the RP2 subgroup, the lipases of the coypu[24] and the guinea-pig[12], were recently found not to show any interfacial activation. Surprisingly, coypu lipase, while not showing any interfacial activation, has a 23 amino acid lid which is homologous to that of the 'classical' pancreatic lipases, whereas the guinea-pig lipase has a mini-lid consisting of only five amino acid residues. Other lipases, such as *Staphylococcus hyicus*, show interfacial activation only with some substrates[25].

'Interfacial activation' of lipases in non-aqueous media

The question of whether lipases can be activated by adsorption onto an interface in organic solvents has been addressed by Klibanov and colleagues[26], using *Rhizomucor miehei* lipase as a model[27]. In aqueous solution, this enzyme was shown to undergo a marked interfacial activation. However, lipase (either lyophilized or precipitated from water with acetone) suspended in ethanol or 2-(2-ethoxyethoxy)ethanol containing triolein exhibited no jump in catalytic activity when the concentration of triolein exceeded its solubility in these solvents, thereby resulting in formation of an interface. To test whether the lack of interfacial activation was due to the insolubility of the enzyme in organic media, lipase was covalently modified with poly(ethylene glycol). The modified lipase, although soluble in non-aqueous media, was still unable to undergo interfacial activation, regardless of the hydrophobicity of the interface. This inability was found to be caused by the absence of adsorption of lipase onto interfaces in organic solvents, presumably because of the absence of the hydrophobic effect (the driving force of lipase adsorption onto hydrophobic interfaces in water) in such media. This lack of interfacial adsorption and activation suggests that the 'lid' covering the active centre of the lipase remains predominantly closed in non-aqueous media, thus contributing to diminished enzymatic activity[26].

Is 'interfacial activation' a criterion for defining lipases?

In conclusion, one can argue that molecular explanations for the phenomenon of interfacial activation need to be looked for not only at the level of the lipase 3D structure but also in the dynamics of organized multimolecular structures and in the interfacial conformations ('interfacial quality') of lipids used as lipase substrates. Among other physicochemical parameters, one can imagine that the radius of curvature of the 'super-substrates' (S_2, S_3 and S_4) (Fig. 1) could play an important role during the interfacial docking of lipases and hence modulate the catalytic activity, through subtle conformational changes.

Interfacial activation as well as the presence of a lid domain are therefore unsuitable criteria on which to determine whether a specific esterase belongs to the lipase sub-family. Interfacial activation has sometimes been wrongly taken as a criterion for predicting the existence of a lid domain in lipases with an unknown 3D structure!

Because naturally occurring lipids are by definition insoluble in water, interfacial activation can be said, in the light of the above overview, to be little more than an artifact which has stimulated the imagination of many biochemists but which has not turned out to be of any very great physiological significance. Lipases might therefore be pragmatically redefined as carboxyl-esterases that catalyse the hydrolysis of long-chain acylglycerols. In fact, they are simply fat-splitting 'ferments'.

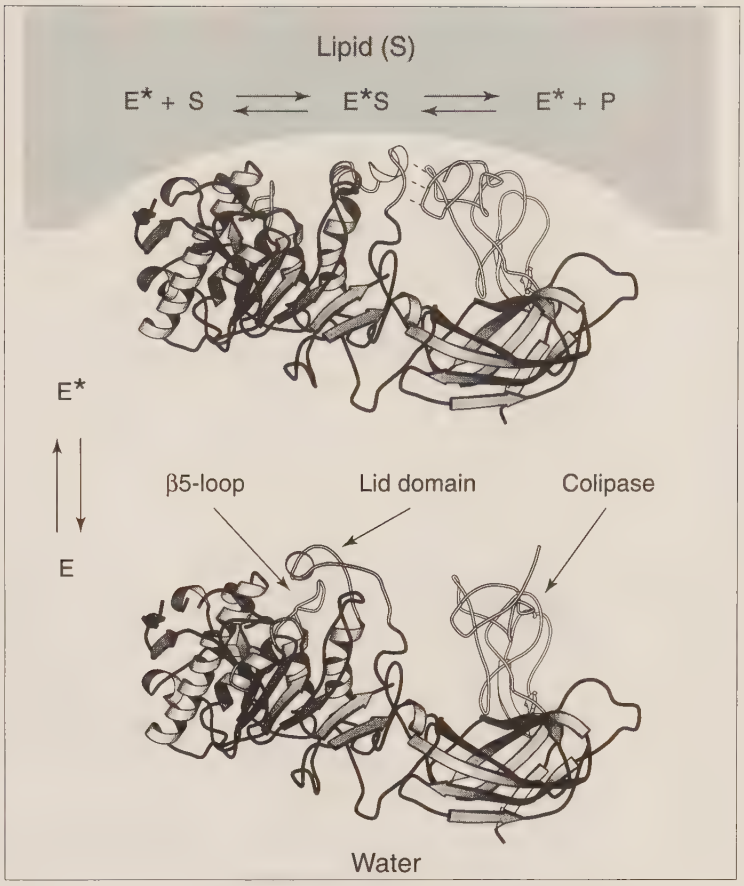

Figure 6

Structure of the HPL–procolipase complex in the closed conformation (E) and in the open conformation (E*S). These two diagrams show the conformational changes in the lid, the β5-loop and the colipase during interfacial activation. Adapted from van Tilbeurgh *et al.*[16,17].

Acknowledgements

I wish to thank F. Ferrato for performing the original experimental work presented in the Figs 3, 4 and 5, as well as L. Sarda and F. Carrière for stimulating discussions. Research was carried out with financial support from the BRIDGE-T-Lipase (Contract BIOT-CT 91-0274 DTEE) and the BIOTECH G-lipase (contracts BIO2-CT94-3041 and BIO2-CT94-3013) programs of the European Union as well as the CNRS-IMABIO project.

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Combinatorial Libraries: Synthesis, Screening and Application Potential

edited by Riccardo Cortese, de Gruyter, 1995. DM138.00 (232 pages)
ISBN 3 11 014395 X

Creating your own biologically active molecule from scratch is a highly demanding procedure. If you are lucky, structures from related compounds may be used as a template to start out from. Systematic alterations followed by testing of individual mutants may eventually lead to the molecule you were looking for.

If, as is most often the case, there is no a priori knowledge of structure–activity relationships between, for instance, a ligand and receptor, the only practical approach is to test a multitude of compounds for the desired activity. Methods for the generation and systematic screening of immense molecular diversities have recently been developed. The common underlying principle of these techniques is based on the generation of expansive collections of very large numbers of molecules (peptides, oligonucleotides or proteins), one or several of which may possess the desired characteristics. Screening and selection isolates the molecules with the desired properties from these combinatorial libraries.

Three basic formats can be distinguished. Synthetic peptide libraries, which may consist of large collections of chemically prepared peptide-chains, either in solution or on beads. Systematic screening identifies the peptide with the desired characteristics. Nucleic acid libraries are constructed from collections of RNA or DNA oligonucleotides; as random oligonucleotides can assume a large number of tertiary structures, some of them may possess desirable binding or catalytic activity against a target molecule. Rounds of selection and PCR amplification yields a pool of molecules enriched in the desired properties. In a molecular biology approach, peptides or whole protein domains are presented on the surface of filamentous phage particles containing in their phage coat the genetic information encoding the presented structures. Phage particles selected for binding to an epitope are propagated in *Escherichia coli* bacteria. With all these techniques, the structure of the tested compound is typically not determined until its biological relevance has been established.

Combinatoral library technology is still at a very early stage of development and thus the relative merits of the different approaches are assessed mostly at a theoretical level. Riccardo Cortese's volume does present ample examples of all the variants of combinatorial library technology, written by scientists who are among the best in their area of research. Applications discussed range from selecting antifungal peptides, thrombin-binding DNA oligonucleotides, antibody epitopes, MHC binding motifs and complete high-affinity antibodies. In each case, the theoretical background, advantages and disadvantages, experimental set-up and results are extensively discussed.

The volume does not provide you with any practical protocols on how to actually perform your experiments, which is rather a pity because success in these types of experiments tends to be highly dependent on tiny details that are easily overlooked by someone not an expert in the field. You will have to work your way through the long lists of references to find these details.

In conclusion, while not providing you with the protocols to start doing your own selections right away, this book will give you a pretty good insight into the possibilities of combinatorial library technology.

John de Kruif
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A golden thread

The Thread of Life: the Story of Genes and Genetic Engineering

by Susan Aldridge, Cambridge University Press, 1996. UK£16.95
(vii + 258 pages) ISBN 0 521 46542 7

This is just the book to throw at your Auntie Madge if she starts asking you to explain what DNA is and whether the scientific community is capable of cloning a human being yet. I would recommend it as an invaluable introduction to genetics, including applications and implications, for anyone developing an interest in the subject. This may include A-level students, undergraduates wanting a very basic introduction before embarking upon a related course, or even the general public.

Susan Aldridge guides her audience through a complex subject with skill, making the basic concepts accessible without compromising accuracy. She provides a historical backdrop for the important discoveries and also tackles the ethical implications of applying new technologies developed from them. Her illustrations are clear and aid explanations of difficult points, but unfortunately lack the innovative style of the text. The book is well structured, dividing the subject matter into manageable chunks with intriguing sub-headings to

retain the reader's interest. This is also achieved by a host of real-life examples, which not only simplify explanation but also act as a constant reminder of the relevance to everyday life of the subject being handled.

The first section of the book answers the question 'what is DNA?'. It starts with a full description of the structure of DNA and relates this to its function via the Central Dogma, pointing out some important exceptions to the rule such as retroviruses and prions. It then puts DNA back into the context of the genome, explaining where DNA is located, how it is packaged and expressed and how genome mapping works. Attention is also drawn to the C-value paradox and the fluidity of the genome. Finally, the author tackles the question of where DNA may have come from in the first place and how it has evolved since then.

The next two sections are concerned with genetic engineering and biotechnology. The former discusses engineering genes: how this can be done, what effects this might have and whether we should be allowed to do it. It covers such areas as DNA fingerprinting, the creation of transgenic animals and the potential of gene therapy. The latter section discusses biotechnology and how it can be utilized in areas as diverse as medicine, the food industry, crop breeding and environmental clean-up programs. In this part of the book there is an emphasis on assessing the acceptability of these ideas to the general public and how controls may be implemented, including a section on patent protection.

The final section briefly assesses whether a full knowledge of molecular genetics can possibly lead us to a comprehensive understanding of nature. It discusses the reductionist view whereby DNA is central to unlocking the elusive secrets of nature. These are countered with some controversial theories such as chaos theory which may have important implications on the matter.

Overall, Susan Aldridge has succeeded in the ambitious task of giving a comprehensive introduction to a fascinating area. She has related subjects of general interest such as gene therapy down to their molecular roots in a way that even Auntie Madge can understand.

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Technology evolution

Immunoassay Automation: An Updated Guide to Systems

edited by Daniel Chan, Academic Press, 1995. US$59.95 (312 pages)
ISBN 0 12 167640 4

The book is an update of the practical guide published in 1992 and provides the reader with a comprehensive review of the new generation of immunoassay systems that have been introduced since the original publication. As Daniel Chan indicates in his introductory chapters, the clinical laboratory environment is changing as a result of pressures on healthcare expenditure, the maturing of the market sector and advances in information technology. This has resulted in the evolution of integrated systems that incorporate the features of different immunoassay systems.

Unlike the previous book, the update does not contain the useful general sections on the important factors to consider when evaluating the technical, clinical, operational and economic performance of an automated immunoassay system. Someone new to the area would be well advised to read the update in conjunction with the original publication. The individual chapters do, however, provide a comprehensive review of the detailed technical features of each of the systems described. The sections give sufficient detail on the technology and its performance to allow meaningful comparisons. To the target audience in the clinical laboratory considering the purchase of a new automated system or those in the industry attempting to keep up with the developments in the area, the review is a useful reference book.

The final three chapters on technologies that may have application are a little incongruous with the rest of the book. In the opinion of the reviewer, these do not provide a comprehensive review of the new technologies that are most likely to have a major impact on the area of future automated immunoassay systems.

Keith May
Unipath Ltd, Norse Road, Bedford, UK MK41 0QG.

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Please send book details of interest to biotechnologists to *TIBTECH* Book Reviews, ETJ, 68 Hills Road, Cambridge,UK CB2 1LA.

Meeting report

Applied antibody technology

Antibodies are proteins which bind specifically to particular antigens, often with high affinity. The specificity and antigen-binding properties have been used since the start of the century, but the range of application has grown rapidly in the last 20 years. The advent of monoclonal antibodies and, more recently, combinatorial antibody libraries displayed on the surface of phage (reviewed by Hoogenboom in this issue of *TIBTECH*), promises a new era for the use of antibodies in diverse fields of application.

Recently, a meeting organized by the Gesellschaft für Biotechnologische Forschung (GBF), Braunschweig, Germany, brought together scientists applying antibody technology to health and environmental problems*.

Antibody structure

The crystal structure of a number of antibody molecules has now been solved. This makes it possible to rationally design purpose-tailored antibodies against antigens of choice. Arne Skerra and co-workers (Technische Hochschule Darmstadt, Germany) have used the canonical backbone structure of an existing antibody as the starting point to create an artificial binding pocket by making defined changes to amino acid side chains within the antigen binding domain. The results of their work show that subtle changes within the structure may play a vital role in antigen binding. It was suggested by Andreas Plückthun (University of Zürich, Switzerland) that such changes may be less than the resolution of a crystal structure.

Display technology

Many laboratories are involved in constructing libraries for the display of a variety of molecules, from peptides to more complex species. The possibility of fusing peptides to the major coat protein pVIII of filamentous phage was explored by George Smith (University of Missouri, Columbia, MO, USA). Riccardo Cortese (IRBM, Pomezia, Italy) and co-workers used peptide libraries to identify unknown disease-specific antibodies from patients' antisera.

The range of libraries being constructed by various meeting participants ranged from fully synthetic (Joachim Wölle, MorphoSys, Munich, Germany) to multi-combinatorial (Regis Sodoyer, Pasteur Merieux Serums and Vaccines, Marcy l'Étoile, France). To reduce the probability of selecting non-specific binders, attempts are being made to make antigen binding a requirement for phage infectivity [Andreas Plückthun, and Andrew Bradbury (Scuola Intern. Superiore di Studi Avancati, Trieste, Italy)]. A threshold affinity between binder and ligand is required to retain infectivity of the phage.

It became evident that different sources for libraries are being sought. Camel antibodies are of special interest as the 'V_{HH}' molecules do not associate with a light chain (Serge Muyldermans, Institute of Biotechnology, Brussels, Belgium). Sheep antibody libraries are being developed in attempts to produce high-affinity binders.

Peptide libraries have been used to identify novel domain-containing proteins such as proteins involved in signal transduction that contain the SH3 domain. Peptide ligands were identified from the phage library and then in turn used to identify novel proteins containing a similar domain (Brian Kay, University of North Carolina, Chapel Hill, NC, USA).

Expression of recombinant antibodies

To make recombinant antibodies a worthwhile alternative to conventional antibodies, a cheap, large-scale method for their production is required. Different expression systems have been studied. These range from bacteria, insect cells and plants to transgenic animals. Each system has its own advantages and problems. In general, the application required governs the expression system used. Problems such as low expression levels of the correctly folded protein in bacterial cells, or lack of post-translational modifications, may demand a change of system. In addition, different vector systems have been designed for the bacterial expression of recombinant proteins potentially toxic to the *Escherichia coli* cells.

Plant-based expression systems present a cost-effective means for the production of recombinant proteins. It is possible to produce whole Fab and IgG molecules in a functional form in various organs of the plant, e.g. seeds or tubers (U. Fiedler and U. Conrad, Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany). Ann Depicker (University of Gent, Gent, Belgium) reported that the signal sequence incorporated into the DNA construct determines the level of accumulation of the recombinant protein (0.02–5% of total protein).

The production of antibody-like proteins was also described for insect cells (Bernd Haase and co-workers, GBF, Braunschweig) and even in live silk worm larvae (Prabhakara Choudary, University of California, Davis, CA, USA).

It would seem that each recombinant antibody sequence behaves differently and expression protocols require optimization on an individual basis.

Applications in health

Anthony Williamson and co-workers (Scripps Research Institute, La Jolla, CA, USA) have selected antivirus antibodies recognizing, among others, HSV, HIV and rubella from human antibody libraries. Other potential infectious agents such as PrP (thought to be involved in the transmission of neurodegenerative disorders) have been targeted. The potential of the technique for use in diagnostics was therefore demonstrated.

Examples of how human monoclonals are being used for the diagnosis and treatment of viral diseases in man, for example CMV and EBV, were presented by A. Lang and co-workers (Swiss Serum and Vaccine Unit, Berne, Switzerland) and Thomas Böldicke (GBF, Braunschweig).

Transgenic mice carrying human κ and heavy chain genes instead of the mouse equivalents were used as an alternative source for human monoclonal antibodies by Nils Lonberg (GenPharm International, Mountain View, CA, USA).

An approach to finding novel tumour markers was described by Simon Hufton (CESAME, University Hospital, Maastricht, The Netherlands). Selection was performed on patient material, in this case colorectal cancer cells lines. Libraries used included cDNA libraries constructed from patient

*The meeting 'Antibody Technology and Applications in Health and Environment' was organized by the Gesellschaft für Biotechnologische Forschung, and was held in Braunschweig, Germany, 6–10 September 1996.

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PII: S0167-7799(96)30027-9

tumours that were then screened against patient sera.

Antibodies against known tumour antigens are already being used in clinical trials and methods for imaging were described by Anna Wu (Beckman Research Institute, Duarte, CA, USA). Anti-CEA molecules were radiolabelled and used for pre- and post-operative tumour imaging with encouraging results for future trials.

Bispecific antibody molecules combining affinity for T cells and for tumour cells were described for the activation and redirection of T cells to targeted tumour cells (Horst Lindhofer, Institut für Immunologie, Munich, Germany). Antibodies have also been used to deliver prodrugs or toxins to the tumour, for treatment of minimal residual disease (Walter Fiers and co-workers, Gent University, Gent, Belgium and Winifried Wels and co-workers, Tumour Biology Centre, Freiburg, Germany).

This approach was also used for the treatment and diagnosis of autoimmune diseases, including inflammatory joint diseases such as rheumatoid arthritis, with anti-CD4 antibodies (Frank Emmrich, University of Leipzig, Leipzig, Germany).

Applications in agriculture

Because the use of hormones in agricultural animals is not acceptable, alternatives in the form of antibodies that mimic hormones, enhance hormone activity or have cytotoxic activity are being sought. An example of an antibody with cytotoxic activity was given by David Flint (Hannah Research Institute, Ayr, UK). He described the use of anti-adipocyte antibodies which bind to, and then destroy, fat cells. This so-called 'immunoliposuction' may be employed to produce leaner animals for human food production.

To reduce the amount of chemicals used in crop production, recombinant antibodies may provide a convenient alternative way of making plants resistant to viruses. Transgenic plants carrying anti-beet necrotic yellow vein virus antibodies were shown to contain reduced levels of virus as compared with wild-type plants, when challenged with virus, under laboratory conditions (Lothar Fecker, Federal Biological Research Centre, Braunschweig). Several groups reported the use of recombinant antibodies for the detection of a variety of plant viruses.

Applications in the environment

Antibodies are also being used to monitor the biodiversity of populations of microorganisms insoils and sediments, as described by Michael Tesar and co-workers (GBF, Braunschweig) and Michael Schloter (Institut für Bodenökologie, Oberschleissheim, Germany).

Removal of organic pollutants (pesticides and heavy metals) from the environment, for example from rivers and drinking water sources, is becoming increasingly important. The use of recombinant antibodies for this task was outlined in detail by Bill Harris and co-workers (University of Aberdeen, Aberdeen, UK) and Karl Kramer (Technical University of Munich, Munich, Germany). Harris was successful in removing organic pollutants present in solution, using high-affinity paraquat or atrazine-binding antibody fragments immobilized on beads. These antibody fragments have also been expressed in transgenic tobacco plants.

Again, for these applications, cost and effectiveness and large-scale production is crucial and, therefore, recombinant antibody technology would seem suitable for the task.

The meeting provided a successful platform for discussing applications of recombinant antibody technology to a very diverse range of subjects. The GBF agreed to host another similar meeting in two years' time.

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Meeting report

Gene to screen

Over the last decade the research and development expenditure of most major pharmaceutical companies has increased 5–10-fold, but there is still an overwhelming demand for better therapies to treat most major diseases. Two areas of current interest and expansion in pharmaceutical research are target identification and diversity of compound collections. A recent conference* was aimed at addressing the former, with a focus on the impact of the human genome project on the discovery of novel molecular targets and pathways for therapeutic intervention. The major themes were:

- Genetic approaches to identify disease susceptibility genes and pathways.
- Transcript profiling (cDNA abundance) in normal and disease tissue.

*The meeting 'The First European Forum on Current Technologies and Strategies to Identify Drug Targets', organized by IBC, was held in London, 14–15 October 1996.

Genetics-based approaches for target identification

In the opening keynote presentation, Jonathan Knowles (Glaxo Wellcome, Geneva, Switzerland) used the example of the *Haemophilus influenzae* sequencing project to illustrate the increase in number of therapeutic approaches as a result of genome sequencing and data analysis. He went on to predict a substantial increase in the number of potential drug targets as we progress towards completion of the human genome sequencing programme in 2002. Moving on to genetics and using Alzheimer's disease and apolipoprotein E as an example, he introduced the concept of a genetic component for most major polygenic diseases. Several subsequent speakers elaborated on the identification of susceptibility genes. Tony Merriman (Wellcome Trust Centre for Human Genetics, Oxford, UK) described the identification of a type 1 diabetes susceptibility locus named *IDDM6*. Following affected sib pair analysis to obtain a linkage interval of approximately 10 000 kb, linkage disequilibrium was then necessary to reduce this to approximately 1000 kb, and it was suggested that this will be a prerequisite for most similar analyses. A two-pronged approach is now being applied towards the identification of the *IDDM6* gene. Merriman and Todd *et al.* are using various gene hunting techniques to identify all genes within this 1000 kb region whereas their collaborators at Merck are sequencing the entire region. Another sequence-based approach, exploiting the synteny between fugu (Japanese puffer fish) and the human genome, was described by Ian Purvis (Glaxo Wellcome, Stevenage, UK). Using their published work on Chr 14 and the Alzheimer's locus as an

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example, he described how the fugu genome, which is 7–8-fold smaller than the human but with highly conserved regions between the two species, requires a significantly reduced sequencing effort. However, two areas of caution were noted. When novel fugu genes show little sequence homology to man, it is very difficult to progress towards the identification of the equivalent human gene. Similarly, problems with missing or significantly different members of a gene family lead to dead ends.

Leena Peltonen (University of Helsinki, Helsinki, Finland) emphasized the advantages of using isolated populations for the analysis of simple as well as complex genetic diseases. Her arguments were based mainly on the assumption that isolated populations simplify complex genetic variations by inheriting reduced chromosomal diversity from their common ancestors. She gave examples where disorders like bipolar disease and schizophrenia may well be segregating in some selected populations in Finland. Jacqui Beckmann (Genethon, France) re-emphasized the power of isolated populations, quoting as an example the use of the Amish community for studies on limb girdle muscular dystrophy. However, one should not presume genetic homogeneity in these isolates, as multiple mutations have been observed, e.g. six LGMD2A haplotypes. This may be due to a digenic inheritance where the founder effect could be on a second, as yet unidentified, locus! He extrapolated from this to say that many so-called monogenic diseases may in fact be digenic or even polygenic. By contrast, he speculated that many polygenic diseases may not be as complex as they first appear.

Zeneca's alliance with the Wellcome Trust Centre for Human Genetics was used to illustrate their approach. The current focus is on rheumatoid arthritis and osteoarthritis, and the majority of the work in the initial phases, including genotyping and genetic analysis, focuses on the partner. Zeneca's effort and resources are directed towards integrating a combination of gene hunting technologies, genome databases and proprietary expression databases (Incyte databases) to find most of the expressed genes from targeted, genetically linked regions. Confirmation of these genes is made via mutation detection and sequencing coupled to target validation biology.

Geoffrey Duyk (Millennium Pharmaceuticals, Cambridge, MA, USA) gave the last talk on genetic approaches to dissecting polygenic diseases. Overall, programmes in fifteen disease areas are in progress though many of these are currently at the sample collection stage. Collections are being carried out worldwide with a focus on sib pair families rather than just sib pairs. Work on obesity was discussed in which, in addition to published animal model studies, an approach to human genetic analysis is being made via a sample collection in collaboration with an organization called TOPS (Take Off Pounds Sensibly) in the USA. In an effort to use the power of discordant sib pairs, they are also aiming to collect 100 sib pairs from China. This is a difficult task since their aim is to collect sib pairs of whom one sibling is at the 95th percentile whereas the other is at the 5th percentile of the weight distribution index of the target population.

Transcript analysis for target identification

The focus then moved to gene expression in diseased and healthy tissue, and David Bailey (Pfizer, Sandwich, Kent, UK) described Pfizer's approach based on the use of public and proprietary databases (Incyte databases). Using prostate hyperplasia as an example, he discussed expression profiles (electronic northerns) as a means of understanding physiological changes within the disease state for symptomatic relief type approaches. He then described the use of these databases as a decision-making tool. Following identification of members of gene families as targets, their compatibility with Pfizer's biological expertise, complexity and bias of compound collection and previously identified chemical leads is assessed. This method of target selection for drug discovery helps to reduce the risk of failure.

The only other talk in this area of gene discovery was presented by Michael Browne (SmithKline Beecham, Harlow, Essex, UK). Following a brief description of the Human Genome Sciences (HGS) database, he presented examples where this had been of real use in drug discovery research. The first application was in the area of lipoprotein associated phospholipase A2 overexpression implicated in atherosclerosis. Efforts aimed at cloning this particular gene were superseded by a hit in the HGS database soon after SB signed up with them. This project has progressed rapidly through the high throughput screening mode and into animal models. An example of homology searching was given in which MCP-4, a member of the chemokine family, was identified, expressed and used in assays that were already in place. Browne also briefly described the discovery of a novel cathepsin from an osteoclast library and its potential implication in osteoporosis. Finally, a link was made back to genetic approaches with a description of how large genomic clones like YACs and BACs can be used as probes against gridded cDNA libraries to derive region-specific cDNA clones.

Gene function

Arthur Sands (Lexicon Genetics, The Woodlands, TX, USA) presented the first of two talks aimed specifically at attempts to understand gene function. Lexicon, a recently established company, aims to produce a clone bank of mouse embryonic stem (ES) cells containing up to 500 000 individual gene knockouts. Given that there are approximately 100 000 genes in the human genome, this bank will represent a five hit complexity covering specific allelic knockouts. Their proposed timelines are 5000 genes knocked out in year 1, increasing to 40 000 knockouts in year 2 and 100 000 knockouts in year 3 and so on, reaching a total of 500 000 by the end of year 5. These extrapolations are based on their success in producing about 50 lines in the first nine months of operation. In another talk on gene function, Paul Bartel (Myriad Genetics, Salt Lake City, UT, USA) discussed yeast hybrid systems for gene function analysis. A test case used the protein interactions map of bacteriophage T7 and managed to detect most interactions. Reasons for the inability to detect all interactions include toxicity in *E. coli*, clone instability in yeast and occlusion of binding sites. False positives were mainly attributed to transcription activation without a protein–protein interaction.

Other related talks

Finally, in a couple of overview talks, Andrew Lyall (Glaxo Wellcome Research) summarized the impact of various aspects of bioinformatics from databases through to

integrated knowledge query tools in helping biologists understand the function of unknown genes and identify suitable targets for drug discovery. The final talk by Murray Tait (Amrad Natural Products, Australia) re-emphasized the need for increased chemical diversity to match the improvements in target identification. He finished with a thought provoking question – should industry embrace the complexities of natural products, combinatorial chemistry, large compound collections, etc., or should choices be made up front? He had no conclusive answers but provided food for thought for the journey home.

Concluding remarks

This conference was designed around strategies and approaches aimed at the identification of drug targets, using the wealth of information coming out of the human and other genome projects. There was adequate detail to make the subsequent transition to drug discovery and, consequently, most of the delegates were comfortable with the content. Hopefully, some went home with ideas that may translate into better health care in the next decade or so!

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TIBTECH Editorial Policy

Trends in Biotechnology is a news, reviews and commentary journal designed to keep its international readership up-to-date with the current developments in biotechnology. *TIBTECH* occupies a niche between primary research journals and conventional review journals – it is not a vehicle for the publication of original research data or methods.

There is strong emphasis on an integrated approach to communicating significant new advances in biotechnology, and discussing their commercial potential. This necessitates combining essential background with state-of-the-art information to make the topics addressed accessible and valuable to newcomers and experts in the field alike. All articles are subject to peer-review and are prepared to strict standards to ensure clarity, scientific accuracy and readability. Commissioning does not guarantee publication.

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Plants as bioreactors for biopharmaceuticals: regulatory considerations

Lucio Miele

Plants are one of several novel hosts that can be used for the production of recombinant biopharmaceuticals such as cytokines, hormones, monoclonal antibodies, enzymes and vaccines. The novelty of this technology and its wide range of potential applications require an assessment of possible regulatory concerns in the clinical development of plant-derived biopharmaceuticals. General principles extrapolated from experience gained with biotechnology products from other sources can serve as a foundation to develop scientifically sound strategies for the large-scale production and clinical development of safe and effective biopharmaceuticals in plant hosts.

Long before the dawn of modern science, humans were using plants for medicinal purposes and as sources of poisons and drugs of abuse. In addition to oxygen, food, clothing and building materials, plants have provided us with a veritable treasure trove of biologically active substances, which humanity has put to good (and bad) uses throughout its history. Some of the most useful weapons in medicine's arsenal, from omnipresent aspirin to digoxin, morphine and taxol to name a few, are molecules that were originally obtained from plants. Biotechnology is now writing a new chapter in this very old story, by using recombinant DNA technology to produce biological macromolecules in crop plants[1–9] for pharmaceutical applications.

This development has potentially important implications for the large-scale manufacture of biopharmaceuticals, and it presents fascinating challenges for biotechnology scientists in industry and academia, as well as for public health agencies whose mission is to ensure the safety and effectiveness of drug products. Fortunately, a great deal of experience has been accumulated in the manufacture of biopharmaceuticals in animal and bacterial cells, as well as in the genetics and biology of crop plants. Elements from these different disciplines can be brought to bear on the design of optimal production strategies and testing protocols.

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Experience with specific products will show us which concerns have practical relevance, and what are the best strategies to deal with them. Probably, unanticipated issues will emerge during the development of some products. Case by case application of sound scientific and clinical judgment will be needed to solve them. Points to Consider documents issued by the FDA, which can be updated as scientific knowledge and clinical experience accumulate, provide a flexible regulatory approach to novel issues in rapidly evolving areas of pharmaceutical research (Box 1).

Recombinant proteins, such as cytokines, antibodies, vaccines, plasma proteins and enzymes, form the vast majority of biological macromolecules currently under investigation or licensed for pharmaceutical use. These molecules differ in many respects from other, relatively simple, chemical entities that have been traditionally obtained from plants. In general, they are relatively unstable molecules that need to be purified in aqueous solutions and cannot withstand extremes of pH and temperature or prolonged contact with organic solvents. They are often produced in relatively low amounts, which poses potential technical problems for the consistent removal of unwanted impurities. Terminal sterilization of purified products by harsh physical or chemical means is generally not feasible. Determination of the exact structure of purified compounds by crystallographic or spectroscopic means is very cumbersome and not always possible. In addition, even minor differences in post-translational modifications can potentially affect the activity and/or immunogenicity of these molecules. Finally, the biological activity of proteins depends upon correct

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PII: S0167-7799(96)10077-9

Box 1. How to obtain FDA documents

Guidance documents, including Points to Consider and other policy documents, are available from CBER (the Center for Biologics Evaluation and Research) as follows:

- Documents may be obtained by FAX by calling the CBER FAX Information System toll-free in the United States at 1 888 CBER-FAX, or at 301 827 3844.
- Persons with access to the INTERNET may obtain documents using the World Wide Web (WWW) or File Transfer Protocol (FTP). For WWW access, connect to CBER at 'http://www.fda.gov/cber/cberftp/html' or 'ftp://ftp.fda.gov/CBER/'.
- For FTP access, connect to the FDA FTP server, FTP.FDA.GOV (192.73.61.21). The CBER documents are maintained in the 'CBER' subdirectory. Logins with the user name of anonymous are permitted, and user's e-mail address should be sent as the password. The 'READ.ME' file in that subdirectory describes the documents that may be available as an ASCII text file (*.TXT), or a WordPerfect 5.2 of 6.x document (*.w51, wp6) or both.
- Documents may also be obtained by 'bounce-back e-mail'. A list of documents available by e-mail can be obtained by sending a message to 'DOC_LIST@A1.CBER.FDA.GOV'. For each document, send a message to 'documentname@A1.CBER.FDA.GOV'. Documents not available by return e-mail may also be requested from 'CBER_INFO@A1.CBER.FDA.GOV'.
- Finally, written requests may be submitted to the Manufacturers Assistance Staff (HFM-42), Center for Biologics Evaluation and Research, Food and Drug Administration, 1401 Rockville Pike, Rockville, MD 20852-1448, USA. Send one self-addressed adhesive label to assist that office in processing the request.

folding, and the stability of the active 'native' conformation. As a consequence, relatively minor variations in production, purification, storage conditions or formulation may sometimes cause large differences in biological activity by affecting the fraction of correctly folded molecules in the final product. Such differences may be observed despite comparable levels of purity and degradation. These characteristics raise regulatory issues that are often different from those traditionally associated with the purification of simpler drugs from plant material. In this brief overview, I shall concentrate on general principles that can be applied in the regulation of the manufacture and testing of recombinant biological macromolecules produced in plants for pharmaceutical use.

Potential applications and production strategies

Some of the possible pharmaceutical applications of plant biotechnology for which basic proof of concept has been obtained experimentally are summarized in Table 1. The potential clinical uses of plant-derived recombinant products are diverse, ranging from replacement therapy to immunotherapy to edible oral vaccines[1–21].

Two basic strategies have been used to produce recombinant proteins in plant hosts[1–3,7,8,10]. One is based upon the generation of transgenic plants by stable integration of a transgene in the plant genome. The other uses plants as hosts for transient expression by using plant viruses as vectors. Each approach has its advantages and disadvantages. Generating stable transgenic plants is more labor-intensive than generating recombinant virus vectors that can infect wild-type plants, often with high yields of product. On the other hand, transgenic plants do not require inoculation and can be obtained in very large amounts once a stable strain has been generated. One interesting potential application of plant viruses is as epitope delivery systems. These are recombinant plant viruses in which a foreign epitope has been fused to a viral capsid protein. This technology could lead to human vaccines, including oral ones, consisting of whole plant viruses engineered to carry appropriate epitopes as exposed regions of their capsids[7–9].

Table 1. Possible applications of plant biotechnology to biopharmaceuticals

Product class	Examples	Refs
Recombinant proteins	Monoclonal antibodies	1,5,11
	Enzymes	12,13
	Hormones	14,15
	Cytokines	16,17
	Plasma proteins	18
	Vaccines	19–21
Chimeric plant viruses	Vaccines	7–9

A detailed review of the potential regulatory issues connected to all potential pharmaceutical applications of plant-derived recombinant products is well beyond the scope of this article. Some general principles, however, do apply to most, if not all, of these products and would probably form the basis for a review of their manufacture and clinical testing by a regulatory agency. These principles are summarized below.

Genetic stability

The establishment of a reliable and continuous source from which the product can be consistently obtained is highly desirable regardless of the expression strategy. Depending upon the chosen expression system, such sources can take different forms (e.g. master cell banks for cell cultures, seed banks for transgenic plants, virus seed stocks for transient expression systems and founder strains for transgenic animals). Seed banks from transgenic plants would probably need to be reamplified periodically because most seeds cannot be stored indefinitely. Storage conditions would have to be carefully optimized to ensure viability, minimize genetic damage and to prevent contamination by insects, bacteria, fungi, etc. Monitoring the stability of the transgene (e.g. by determining the predominant DNA sequence) and the levels of product expression in representative plants of a given lot, from lot-to-lot and after reamplification of a seed bank

may be advisable. Additional data may be derived by comparing a product made from different seed banks. The main objective of these studies would be to ensure product consistency (e.g. by identifying mutations occurring in the transgene sequence). From a regulatory standpoint, variations in product expression levels are only relevant insofar as they may affect product safety and efficacy (see section on Purity). In the case of virus-based transient expression systems, early concerns about sequence drift have been generally dismissed for tobacco-mosaic-virus-based vectors (and for other RNA-based viral vectors) (see Ref. 10). Similarly, genetic instability with reversion to wild type was initially observed when cowpea mosaic virus (CPMV) was used to express an epitope of the foot and mouth disease virus[22]. This problem was overcome by optimizing the site of insertion into the viral genome[22], and stable recombinant CPMV was obtained that expressed rhinovirus 14 and HIV-1 epitopes[22]. These examples illustrate the need for monitoring and optimizing the genetic stability of each expression system. As for any other biopharmaceutical, a program to monitor lot-to-lot consistency of the biochemical and biological properties of the product by comparison with appropriate in-house reference standards would be an integral part of product development.

Microbiological considerations

In general, the intrinsic instability of biological macromolecules makes it impossible to use terminal sterilization strategies based on harsh physical and/or chemical treatments. In addition, many of the raw materials used to manufacture biological macromolecules can support the growth or survival of potentially harmful organisms. These concerns apply in various measures to virtually all biopharmaceutical macromolecules produced from biological sources. Some of these concerns, particularly in the area of viral safety, are probably less serious for plants than for animal and human sources of raw material. To date, plant viruses are not known to infect humans or animals, nor are they potentially pathogenic human and animal viruses capable of replicating in plant cells. The same cannot be said for bacteria and fungi. Thus, as for all recombinant biopharmaceuticals, the whole manufacturing process, from qualification and storage of raw materials to sterile filling of final containers, should be designed to minimize potential bacterial and fungal contamination, and to ensure reproducible removal of bacteria, fungi and their byproducts during purification. As for any other purified biopharmaceutical, the final product should be sterile and not contain unacceptable levels of bacterial endotoxin.

The manufacturing establishment should be designed with appropriate attention to the flow of personnel and materials, and to water, heating, ventilation and air conditioning systems. Bioburden testing, sterility and endotoxin testing should be conducted at appropriate stages during production (e.g. on unpurified bulk product, purified bulk product and final, filled product). Guidance on the institution of appropriate in-process testing of bacterial and fungal contaminants can be found in several Points to Consider documents issued by the FDA and in International Conference on Harmonisation (ICH) consensus documents[23–26]. In the case of plants, contamination of raw materials by fungi that produce mycotoxins (e.g. *Aspergillus flavus*), by toxigenic soil bacteria (e.g. *Clostridium botulinum*), by endotoxin-releasing Gram-negative bacteria or by other potential pathogens should be prevented or limited as much as possible. Experience gained in the food industry on the control of such contaminants will be of value, particularly for orally administered products. However, it should be noted that for parenterally administered biopharmaceuticals the bioavailability and absorption rate of contaminant toxins would probably be higher than if they were ingested with food, and their effects may be different.

Purity

Plant-derived recombinant products can be purified by standard biochemical techniques, and their purity can be assessed and monitored on a lot-to-lot basis in the same way as for all other biotechnology products. Impurities of potential concern in plant-derived biopharmaceuticals may include biologically active plant metabolites or alkaloids (e.g. nicotine), plant macromolecules (extraneous proteins, DNA, polysaccharides and lipids), pesticides and herbicides. Theoretically, such impurities could affect the safety and efficacy of the product in a number of ways, including: (1) direct toxic effects on the recipient (pesticides, herbicides and plant metabolites); (2) effects on product stability and biodistribution (e.g. polysaccharides binding to a recombinant protein by electrostatic interactions); or (3) immunogenicity leading to sensitization and allergic reactions (e.g. to contaminant plant proteins). The importance of the latter concern, which is not limited to plant-derived products, would depend on the intended use of the biopharmaceutical (single dose versus repeated administration, dose, route of administration, etc.).

Testing protocols and/or validation of the ability of the purification process to remove a given contaminant can be used to monitor removal of potentially harmful contaminants, depending on the nature of the contaminants, the availability of sensitive and rapid detection systems, etc. This raises the issue of variability in product yield. The concept of validating the removal or inactivation of a given contaminant implies that it is possible to estimate: (1) the 'load' of contaminant in the unpurified bulk; and (2) the volume of unpurified bulk product needed to generate a dose of final product. The latter is a function of the concentration of the desired product in the unpurified bulk. The goal of such a validation is to establish, by a statistically acceptable experimental design, that the purification scheme can remove substantially more contaminant than is present in the volume of unpurified bulk required to produce a single maximal dose of the final product.

For plants grown in the field one might expect wider variability of product yield from harvest-to-harvest, field-to-field and plant-to-plant than is generally observed when biopharmaceuticals are manufactured under tightly controlled conditions (e.g. in cell culture systems). Wide variations in the relative concentrations of product and contaminants could exceed the capacity of a given purification scheme to remove or inactivate a contaminant. One possible strategy to solve this problem would be to: (1) determine the concentrations of product and contaminant in the unpurified bulk under different growth conditions; (2) validate one's purification scheme for removal of a given contaminant for a range of product concentrations in the unpurified bulk; and (3) from these data, establish acceptance criteria for downstream processing of unpurified bulk lots. Within a given harvest, it may be advisable to test product from different fields separately to avoid pooling low yield with acceptable yield material. Combining material from fields subjected to different types or levels of chemical treatment need not be proscribed if assays of acceptable sensitivity, specificity and interassay variation are used to test for these contaminants or, whenever applicable, the purification scheme is validated for their removal up to substantially higher levels than observed in the pooled unpurified bulk.

This might be straightforward for pesticides, herbicides or well-known alkaloids and metabolites; however, it might not be easy to decide which, if any, other endogenous contaminant(s) to monitor. Notably, the relatively mild aqueous extraction procedures often used to solubilize recombinant proteins might not be very effective in extracting many potential harmful alkaloids, metabolites or pesticides, compared with the organic solvent treatments that are often employed in the extraction of other pharmaceuticals. By contrast, potentially bioactive, water-soluble impurities (e.g. proteins or glycans) may co-purify with recombinant proteins. The use of extensively characterized plant species may be advantageous in this respect. Well-planned animal toxicology studies conducted during preclinical development would be helpful in determining: (1) whether potentially harmful substances are present in any production intermediates; (2) whether toxic effects correlate with the levels of one or more specific contaminants; and (3) whether these contaminants are adequately removed during downstream processing.

Product comparability

Recombinant macromolecules produced in plants should be biochemically, pharmacologically and clinically comparable to their counterparts produced in traditional cell substrates or animals or purified from human sources, if such products are available. This would be particularly important if plant-produced material were intended to replace animal or human-derived material during late product development or after licensing. The most important structural differences one can expect between plant-derived and animal- or human-derived products are in the glycosylation pattern. Potential concerns related to glycosylation differences between 'natural' and recombinant proteins are not confined to plants, and their importance depends on the specific products being considered. For example, glycosylation in the Fc region rarely affects the immunoreactivity of monoclonal antibodies (mAbs). However, Fc region glycosylation does affect Fc receptor binding and antibody-mediated cytotoxicity, and has been suggested to be involved in antibody elimination[27,28]. Glycosylation of Fab regions has been shown to affect binding activity of some mAbs (Ref. 29) but not others[30]. Glycosylation in the hinge region has been suggested to affect antibody sensitivity to proteolytic degradation[31]. Thus, the relative importance of glycosylation differences in mAbs would depend upon the type of mAb product [intact immunoglobulin versus Fab fragments], the intended indication and expected mechanism of pharmacological activity. In some cases (e.g. tumor imaging agents) a lower affinity for Fc receptors may actually be an advantage. Similarly, changes in glycosylation patterns can potentially affect the pharmacokinetics and activity of several cytokines and hormones that are naturally glycosylated in humans[32–35].

While this does not preclude the clinical development of products with glycosylation patterns different from the 'natural' ones, these factors need to be carefully considered and controlled in individual products. The possibility that patients may develop an immune response to plant-derived oligosaccharide moieties also needs to be taken into account in planning the characterization of the product and in designing exploratory clinical trials (although humans may be tolerant to determinants found in common foodstuffs). Immunogenicity would not necessarily make a product clinically useless. However, its potential effects on pharmacokinetics, optimal dose and product activity (if neutralizing antibodies are produced) need to be kept in mind, as does the possibility of allergic reactions upon repeated administration. Enzymatic removal of unwanted glycan groups or the use of mutant or recombinant plant strains with altered glycosylation pathways[36] are possible ways to approach this problem in those cases in which inappropriate glycosylation affects the pharmacokinetics, immunogenicity or efficacy of a product.

A battery of physical, chemical and biological or immunochemical tests should be instituted for the purpose of establishing comparability between products derived from plants and those from animals or humans[22–24,37]. In the case of recombinant proteins, a combination of SDS-PAGE, IEF, HPLC, mass spectrometry, peptide mapping or other appropriate physico-chemical methods could be used. *In vitro* potency assays can often be used to screen for possible differences in bioactivity. To be useful, such assays would have to be appropriately characterized by determining sensitivity and robustness, as well as intra- and interassay variation. Side-by-side comparisons of production lots with the in-house reference standard

should be routinely performed for physico-chemical analysis and *in vitro* potency assays. When the plant-derived product appears to be different from its animal or human counterpart by biochemical and/or *in vitro* biological criteria, pharmacokinetic studies should, generally, be done. In some cases, such studies can be conducted in relevant animal models. Depending upon the role played by specific receptor or antigen binding in product biodistribution and elimination, it may or may not be advisable to carry out pharmacokinetic studies in animal models expressing specific receptors or antigenic determinants. When no appropriate animal models are available, clinical trials with pharmacokinetic endpoints can be used to assess comparability. If potency differences are observed *in vitro* between plant-derived and animal- or human-derived materials and there are no appropriate animal models in which potency can be tested, comparability may have to be established in clinical trials. If adequate pharmacodynamic endpoints can be used to estimate biological activity (e.g. clinical laboratory parameters) they can be used to assess comparability.

Environmental impact

A priori, there is no reason why a transgenic crop plant expressing a human cytokine or a mAb should pose any more environmental risks than a similar plant engineered to resist parasites or to produce slow-ripening tomatoes. Nevertheless, when treading in uncharted territories, proceeding with caution is in order. Thus, the possibility should be borne in mind of uncontrolled spread of transgenic plants in the environment (e.g. by pollen transfer, by horizontal gene transfer to soil microorganisms or simply because the transgenic plant may have a selective advantage over its wild relatives). It is very difficult to make generalizations in this area but accurate risk assessments should certainly be made and adequate measures should be taken to prevent adverse consequences. This should be kept in mind when choosing plant species, geographic locations of test plots and, eventually, production fields and monitoring protocols. An excellent short review of the current understanding of these issues can be found in Ref. 38. Needless to say, if potential plant pathogens (e.g. viruses) are used as vectors for gene transfer, consideration should be given to appropriate containment measures to prevent infection of crop plants. In some cases, the product itself may present an environmental hazard (e.g. a recombinant toxin may conceivably contaminate the soil or affect wildlife eating the plant). The relevant environmental and agricultural authorities should be consulted during early product development.

Conclusions

Thanks to molecular biology, we now have the ability to use plants as a potentially safe and cost-effective source of recombinant biological macromolecules and vaccines for medical uses. This technology has the capacity for endless applications, and attempting to predict all future concerns or pitfalls would be futile. While some generalizations are possible, each plant species and each particular biopharmaceutical product will present different challenges. These challenges cannot be met just with generalized checklists of tests to perform and measures to take. Combined efforts by industry, academic and government scientists in the fields of agriculture, plant biology, pharmacology and clinical sciences will help us identify potential problems in developing specific biopharmaceuticals for specific indications in transgenic plants. Data from ongoing basic and applied research, as well as experience accumulated with other sources of biopharmaceuticals and in plant biotechnology for non-pharmaceutical uses, will help us find appropriate solutions in individual cases.

Acknowledgements

The author is grateful to Drs Jay Siegel, Katy Stein, Thomas Hoffman, Leslie Shelly, Ennan Guan, R. DeLap, W. J. Schmidt and Chana Fuchs for critical reading of this manuscript.

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Lipid-coated enzymes as efficient catalysts in organic media

Yoshio Okahata and Toshiaki Mori

A new way of solubilizing hydrolytic enzymes so that they can be used in organic solvents is to coat them with lipid monolayers. Lipid-coated enzymes have been used successfully for a number of different types of reactions in organic media or organic–aqueous, two-phase systems; for example, enantioselective esterification by lipase, transphosphatidylation of water-insoluble phospholipids by phospholipase D, hydrolysis of a lipophilic substrate by a catalytic antibody and transglycosylation by β-D-galactosidase.

In recent years there has been much interest in the use of enzymes as catalysts in organic solvents[1–3]. The advantage of using hydrolytic enzymes in organic solvents lies in increasing the solubility of lipophilic substrates and also in causing reverse reactions such as enantioselective esterification or transesterification. There have been several approaches to using a lipase as a synthetic catalyst in organic solvents[4–6]. In addition to the water-in-oil emulsion and the reversed micellar system containing a small amount of water[4], there have been two previous reports of the use of both hydrophobic and hydrophilic organic solvents as a reaction medium for lipase: (1) Klibanov and co-workers reported the direct dispersion of powdered lipase in organic solvents, to produce an ester exchange catalyst for heterogeneous solutions[5]; and (2) Inada and co-workers prepared a poly(ethylene glycol) (PEG)-grafted lipase that is soluble or swells in hydrophobic organic solvents and catalyses simple ester syntheses from aliphatic alcohols and acids[6]. Of prime importance for the use of enzymes in organic media is the need to avoid enzyme deactivation or denaturation.

In this article we introduce a new method of solubilizing enzymes in hydrophobic organic solvents, namely by coating the enzyme with a lipid monolayer (Fig. 1)[7–18]. A lipid-coated enzyme has been prepared simply by mixing aqueous solutions of both enzyme and lipid and collecting and lyophilizing the resulting precipitate. The prepared lipid-coated enzyme was insoluble in buffer solution but freely soluble in most organic solvents such as benzene, ethyl acetate, isooctane, isopropyl ether, dimethyl sulfoxide (DMSO) and ethanol. In the lipid-coated enzyme, the hydrophilic head groups of the lipids interact with the hydrophilic surface of the enzyme and the lipophilic alkyl chains extend away from its surface and solubilize the enzyme in hydrophobic organic solvents. The protein content in the complex was estimated to be 8–10 wt%, that is 150 ± 50 lipid molecules per enzyme molecule, both from elemental analysis (C, H and N) and from the UV absorption measurements of the aromatic amino acid residues in the protein[10,13]. Gel permeation

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PII: S0167-7799(97)01003-2

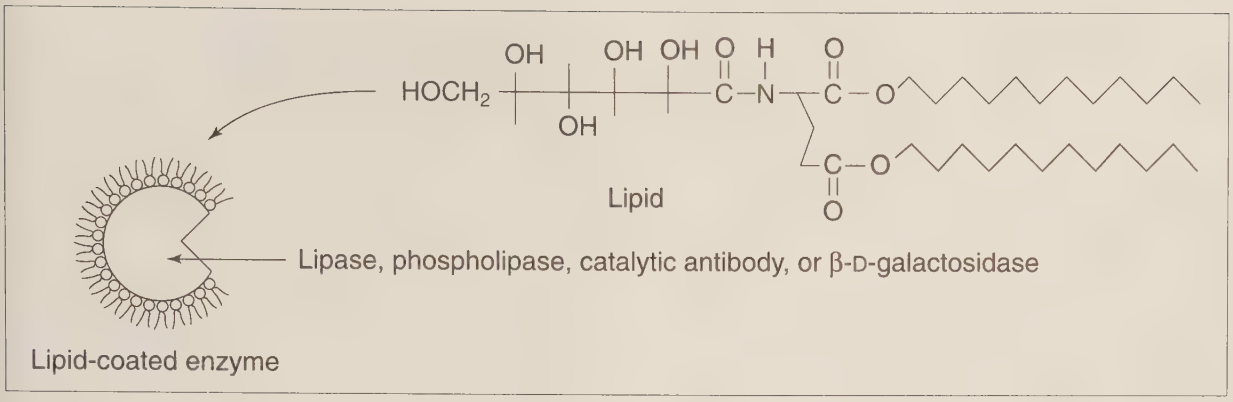

Figure 1
A schematic illustration of lipid-coated enzymes.

chromatography in organic solvents confirmed that the lipid-coated enzyme molecules do not aggregate and that the coating lipids are stable and are not removed from the enzyme surface in organic media[12].

Enantioselective esterification catalysed by a lipid-coated lipase in isooctane

Figure 2 shows typical conversion versus time plots of ester syntheses from (*R*)-, (*S*)-, and racemic 1-phenylethanol and excess lauric acid catalysed by a lipid-coated lipase B (from *Pseudomonas fragi* 22-39B) in dry isooctane at 40°C. When the racemic alcohol was used, the esterification reached equilibrium near 50% conversion, within 2 h. The (*R*)-isomer was completely converted into ester, but the (*S*)-isomer scarcely reacted. This indicates clearly that a lipid-coated lipase can recognize the (*R*)-1-phenylethanol isomer and convert it into ester, but not the (*S*)-isomer.

We compared our lipid-coated lipase system with other systems under the same reaction conditions. The changes in conversion with time of these reactions are shown in Fig. 3. When the lipid-coated lipase B was used (curve a), (*R*)-1-phenylethanol was completely converted into the ester within 3 h with high enantioselectivity [$\nu_R = 50\ \mu M\ s^{-1}$ (mg of protein)$^{-1}$, $\nu_R/\nu_S = 250$]. When lipase powder was dispersed directly in isooctane[5], it gave a very slow reaction rate compared with that of the lipid-coated lipase [$\nu_R = 0.5\ \mu M\ s^{-1}$ (mg of protein)$^{-1}$, $\nu_R/\nu_S = 260$]. In the dispersion method (curve d), the enzyme exists as a suspension in the substrate organic solution, therefore, a large amount of enzyme may be required to achieve a fairly high reaction rate. In the water-in-oil system (curve c)[4], in which 1 mg of native lipase B is solubilized in a buffer solution (pH 5.6) and emulsified in isooctane, the rate of ester synthesis was very slow [$\nu_R < 0.1\ \mu M\ s^{-1}$ (mg of protein)$^{-1}$, $\nu_R/\nu_S = 80$–120] and decreased with increasing reaction time. It seems that the presence of a small amount of water in the water-in-oil emulsion system caused the reverse hydrolysis reaction. When PEG-grafted lipase B was used (curve b)[6], the esterification proceeded at an intermediate rate with high enantioselectivity [$\nu_R = 30\ \mu M\ s^{-1}$ (mg of protein)$^{-1}$, $\nu_R/\nu_S = 280$] in the initial stages. However, the conversion reached a plateau at 70% yield after 40 h in dry isooctane. This suggests that the water produced in the reaction is retained near the amphiphilic PEG chains and causes hydrolysis.

Thus, the lipid-coated lipase can effectively and completely catalyse ester synthesis in dry organic solutions without changing the enzyme enantioselectivity, because unlike the other enzyme systems the lipid-coated lipase is homogeneously soluble and stable in dry organic solvents.

The lipid-coating method can also be applied to phospholipase D (PLD); lipid-coated PLD acts as an efficient catalyst for transphosphatidylation of water-insoluble phospholipids in organic solvents[13].

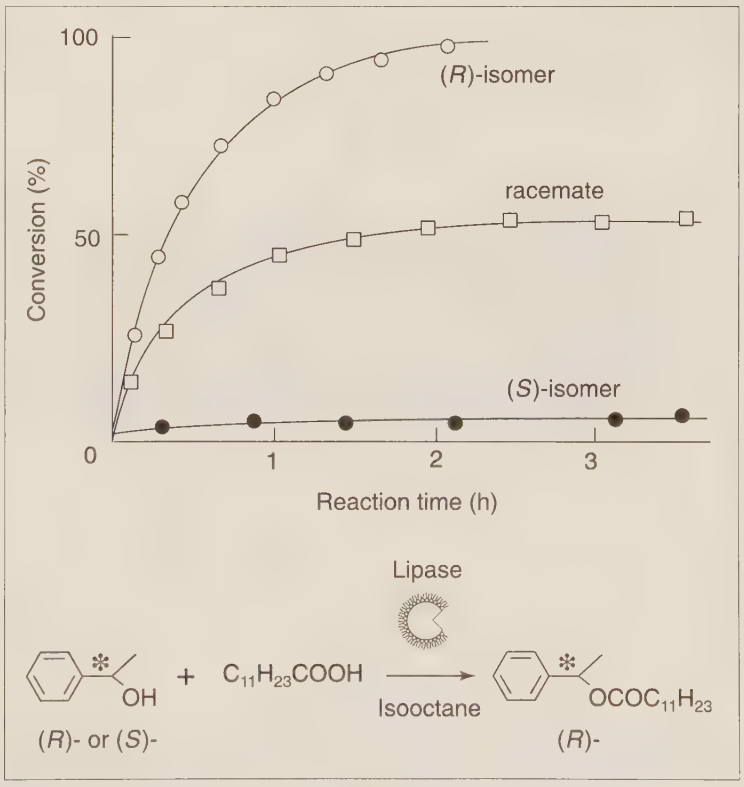

Figure 2
Typical conversion versus time plots of esterification of (*R*)-, (*S*)- or racemic-1-phenylethanol (50 mM) with lauric acid (500 mM) catalysed by a lipid-coated lipase B (1 mg of protein) in dry isooctane (5 ml, H_2O: 60–80 p.p.m.) at 40°C.

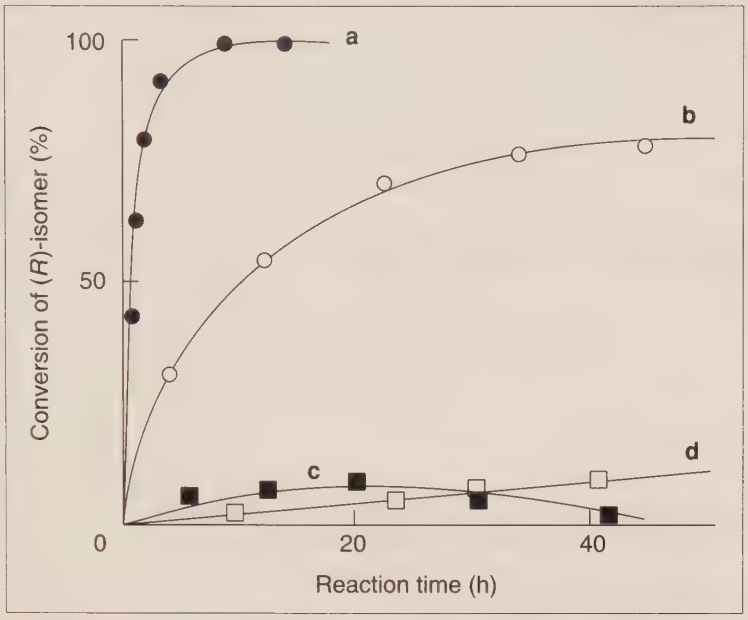

Figure 3

Comparison of catalytic activities for ester synthesis from (*R*)-1-phenylethanol and lauric acid, in dry isooctane at 40°C, catalysed by (**a**) lipid-coated lipase B; (**b**), PEG-grafted lipase B; (**c**), lipase B in water-in-oil emulsion; and (**d**) direct dispersion of lipase B powder. Reaction conditions are as in Fig. 2.

Lipid-coated catalytic antibodies in water-miscible organic solvents

To date, numerous publications have appeared on catalytic antibodies. Various reactions, such as the hydrolysis of esters and amides, transesterifications, Diels–Alder reactions, decarboxylations and sigmatropic rearrangements have been reported to be catalysed by catalytic antibodies[19–23]. Catalytic antibodies are induced by compounds (haptens) that mimic the transition states in these chemical reactions. Although substrates in these reactions are generally lipophilic and not very water-soluble, the reactions have been always carried out in aqueous buffer solutions because catalytic antibodies are thought to be soluble and stable only in aqueous solution.

In an effort to make catalytic antibodies more attractive catalysts from a synthetic standpoint, we applied our lipid-coating method to solubilize catalytic antibodies in water-miscible organic solvents[14]. The catalytic hydrolysis of the monoester of chloramphenicol was chosen as an example because the substrate is lipophilic and the catalytic reaction has been studied in aqueous solution[24,25].

Figure 4 shows the effect of the dimethyl sulfoxide (DMSO) content of the buffer solution on hydrolysis

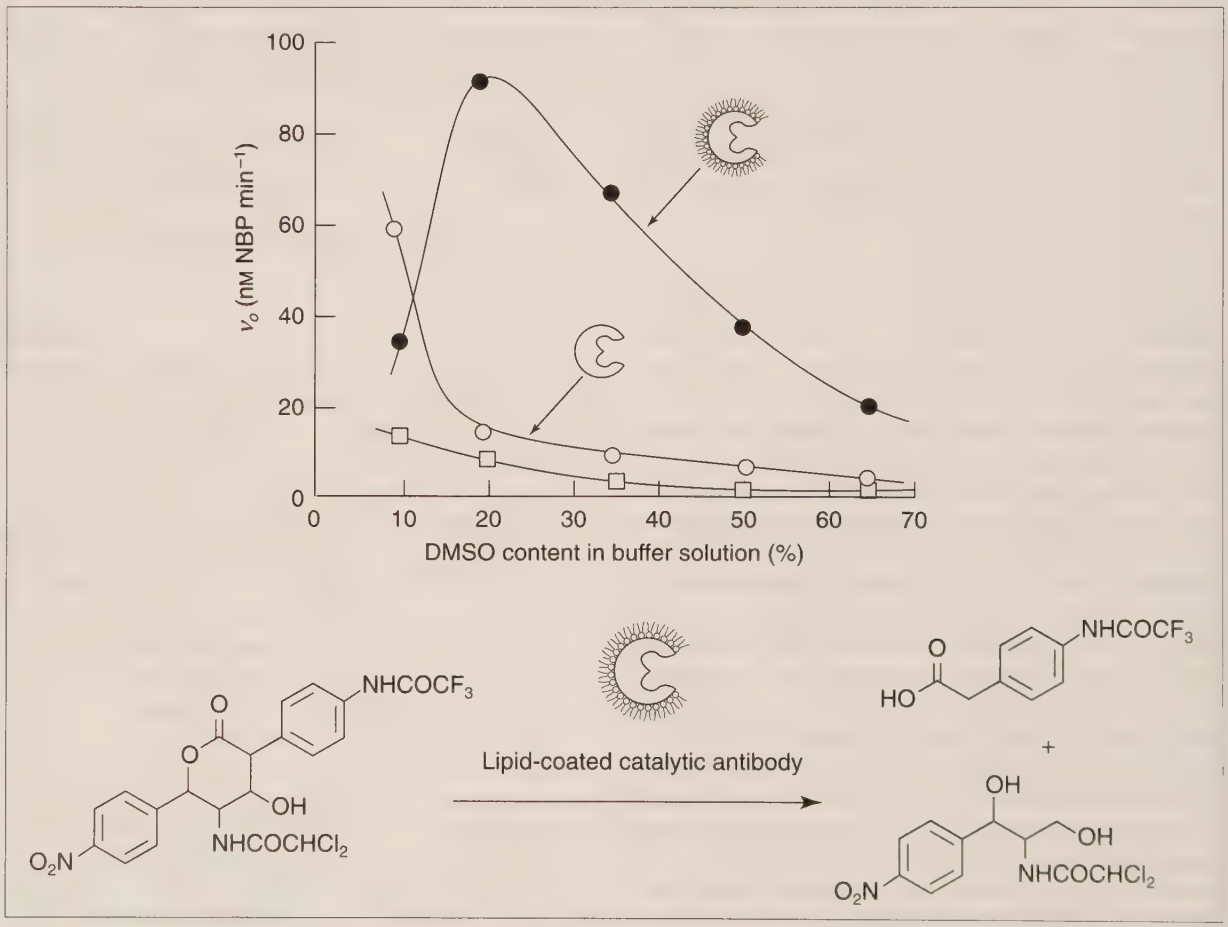

Figure 4

Effect of DMSO content in a Tris buffer solution (0.05 M, pH 8.0) on the hydrolysis of the monoester of chloramphenicol (55 mM) at 30°C. Catalysed by a lipid-coated antibody (1 mM, 30 mg of protein) (●), by a native antibody (1 mM, 30 mg of protein) (○), and spontaneous hydrolysis (□).

rates at 30°C. DMSO must be added to a concentration of at least 20% (by volume) to solubilize the lipophilic substrate completely. At concentrations of between 20 and 65% (by volume) DMSO in buffer solution, both substrates and native or lipid-coated antibodies were homogeneously soluble, but the catalytic activity of the lipid-coated antibody was higher than that of the native antibody because the native species were denatured by DMSO. Similar denaturing was observed for other water-miscible organic solvents such as acetone, acetonitrile and dimethyl-formamide.

Because catalytic antibodies are designed to catalyse a wide range of organic reactions with lipophilic substrates, it is important to use antibodies in water-miscible or hydrophobic organic solvents. The physical stability of an antibody is also expected to be increased by a lipid-coating.

Transgalactosylation catalysed by a lipid-coated β-D-galactosidase in a water–organic two-phase system

In recent years, glycoside hydrolases have been applied as transglycosylation catalysts to synthesize glycoside compounds or oligosaccharides in aqueous solution containing water-miscible organic solvents, by making use of the reverse hydrolysis reaction[26–29]. In contrast to chemical synthesis, enzymatic synthesis has the advantage of providing regio- and stereoselective products in a one-step reaction without the use of protection groups. However, in enzymatic syntheses using glycoside hydrolases it has proved difficult to obtain high yields of glycosylation products because, in aqueous solution, the hydrolysis reaction predominates over transglycosylation[26–29]. If the reaction could be carried out in nonaqueous organic solvents without denaturing the enzymes, high yields of transglycosylation products would be obtained. We have also used our lipid-coating method to solubilize β-D-galactosidase in organic media. As galactosyl donors, such as lactose, are not very soluble in organic media, an organic–water two-phase system was employed, in which both the lipid-coated enzyme and the galactosyl acceptor, an alcohol, are in the organic phase and lactose is in the water phase.

Figure 5 shows typical conversion versus time plots of the transgalactosylation from a tenfold excess of lactose (as a galactosyl donor) to 5-phenylpentan-1-ol ($PhC_5H_{10}OH$) (as a galactosyl acceptor) in a two-phase system of isopropyl ether and phosphate buffer (10 mM, pH 5.1) at 30°C. When the lipid-coated β-D-galactosidase was solubilized in the organic phase (Fig. 5a), a 66% of yield of transglycosylated $Gal\text{-}OC_5H_{10}Ph$ was obtained, but no lactose was hydrolysed even after eight days. It was confirmed by ^1H- and ^{13}C-NMR that the chemical structure of the $Gal\text{-}OC_5H_{10}Ph$ product remained in the β-configuration of D-galactose. As the amount of $PhC_5H_{10}OH$ consumed corresponds to that of $Gal\text{-}OC_5H_{10}Ph$ produced, this means that the carbocation intermediate formed at the active site of the enzyme in isopropyl

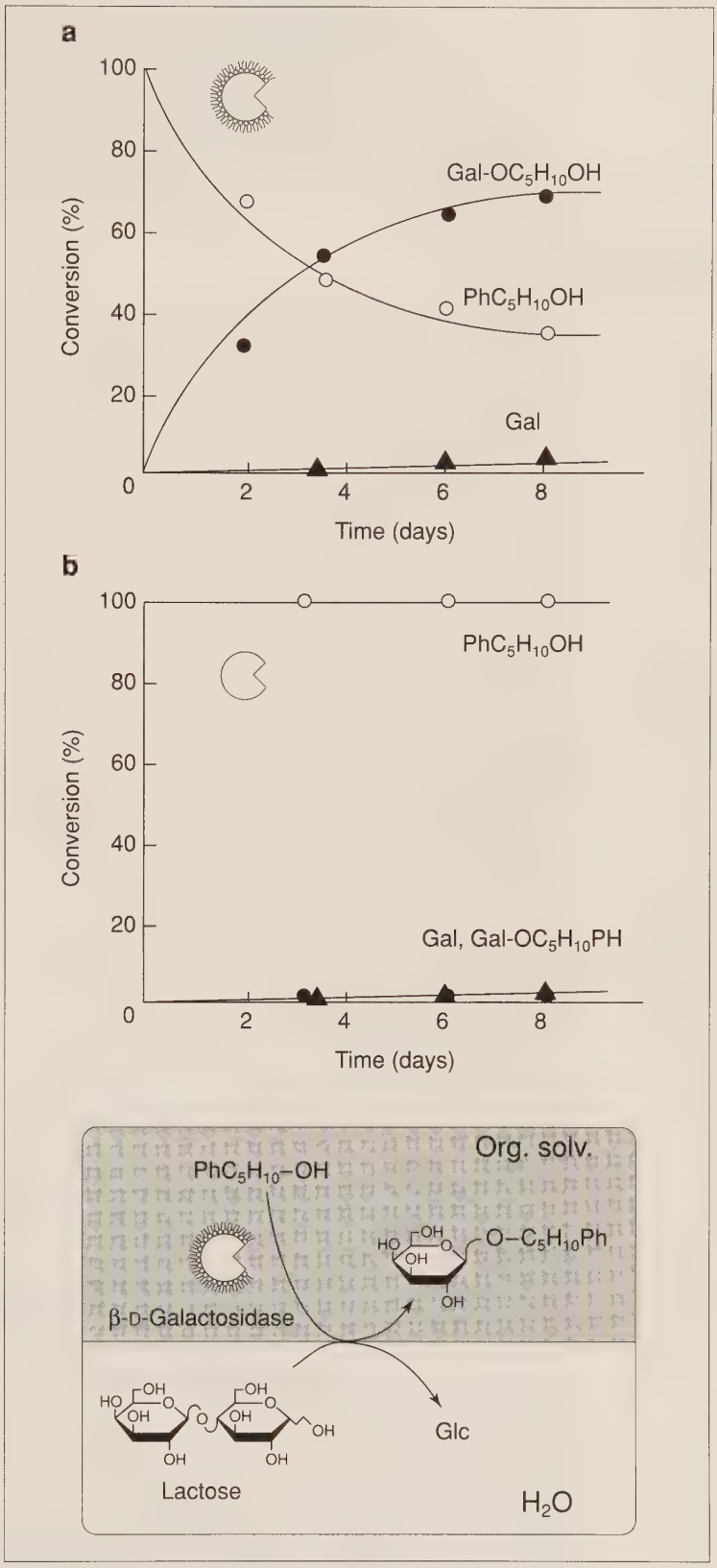

Figure 5

Typical conversion versus time plots of transgalactosylation from lactose (10 mM) to 5-phenylpentan-1-ol ($PhC_5H_{10}OH$, 1.0 mM) at 30°C catalysed by (**a**) a lipid-coated β-D-galactosidase and (**b**) a native β-D-galactosidase from *Escherichia coli* in two phases of isopropyl ether and 10 mM phosphate buffer (pH 5.1). [Enzyme] = 0.1 mg of protein per 20 ml. The lower part of the figure shows the transglycosylation, catalysed by a lipid-coated enzyme, between galactose acceptor alcohols in the organic solution and lactose in the buffer solution.

ether was attacked mainly by the alcohol. By contrast, when a native β-D-galactosidase was employed (Fig. 5b), the starting substrate $PhC_5H_{10}OH$ was completely recovered after eight days. Because neither the galactosylation nor the hydrolysis reaction proceeded in the two-phase system, a native enzyme may be denatured at the interface between the aqueous and organic phases.

Prospects

The lipid-coating system for enzymes described here is useful for solubilizing enzymes in organic media without denaturation. We have shown that this lipid-coating method can be applied to various enzymes as well as catalytic antibodies.

The system has a variety of possibilities for other applications. When the organic solution of the lipid-coated enzyme is cast on an electrode substrate, a thin enzyme film can be obtained as a sensor membrane that is insoluble in aqueous solution[17,18]. When DNA strands were used instead of enzymes, a DNA–lipid complex formed as precipitates, similar to that for an enzyme–lipid complex. The DNA–lipid complex is soluble only in organic solvents and DNAs can form a double strand structure even in chloroform solution[30,31]. We can also prepare a cast film of a DNA–lipid complex that is self-standing, transparent and physically strong. DNA strands in the film can be aligned in one direction by stretching the film, and act as a one-dimensional electron transfer media[30–32].

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Structural studies and synthetic applications of Baeyer–Villiger monooxygenases

Andrew Willetts

Baeyer–Villiger monooxygenases (BVMOs) are enzymes able to perform highly regio- plus stereoselective nucleophilic and electrophilic biooxygenations on various substrates. The resultant chiral products (lactones and sulfoxides) can be valuable for the chemoenzymatic synthesis of a wide range of useful compounds. Recent studies have provided a number of alternative active-site models that attempt to explain the exquisite and unusual selectivity of BVMOs. This article reviews some of the established applications, and considers the merits of the various predictive models.

The Baeyer–Villiger monooxygenases (BVMOs) (EC 1.14.13.x) are an interesting group of flavoproteins with considerable proven ability for yielding key chiral products of value in the chemoenzymatic synthesis of a wide range of useful compounds[1]. These enzymes exhibit the rare characteristic, shared with some other notable biocatalysts such as ribulose bisphosphate carboxylase/oxygenase, of being able to catalyse two mechanistically different types of biochemical reactions, apparently within the confines of the same active site.

BVMOs were first recognized over 40 years ago, during the initial era of steroid biotransformations, as carbonyl monooxygenases (Fig. 1a) with the ability to catalyse the nucleophilic oxygenation of ketones and aldehydes in a manner analogous to the established peracid-catalysed organic chemical reaction from which they take their name[3]. However, more recently it has been recognized that these enzymes can also catalyse the electrophilic oxygenation of various heteroatoms, as illustrated by their ability to form sulfoxides from organosulfides[4]. Both types of reaction can proceed with exquisite selectivity. For example, cyclohexanone monooxygenase (CHMO) purified from cyclohexanol-grown *Acinetobacter calcoaceticus* (NCIMB 9871) yields homochiral (*S*)-5-methyl-(*E*)-lactone [enantiomeric excess (e.e.) = 98%] from 4-methylcyclohexanone[5], and (*R*)-methyl phenyl sulfoxide (e.e. = 99%) from the equivalent sulfide[6].

BVMOs in nature

Almost without exception, the enzymes studied to date have been found in microorganisms, including bacteria of the genera *Acinetobacter*, *Pseudomonas*, *Xanthobacter*, *Rhodococcus* and *Nocardia*, and fungi of the genera *Curvularia*, *Dreschlera*, *Exophilia*, *Cunninghamella* and *Cylindrocarpon*. In bacteria, the natural role of these enzymes appears to be to catalyse one or more key steps in various oxidative catabolic pathways[7], whereas in fungi they appear to play some, as yet, poorly characterized role in the programme of biochemical events that accompanies the switch from primary to secondary metabolism. When, as in the case of cyclohexanol-grown *A. calcoaceticus*, there is only one induced BVMO present in the microbial cells, it is possible to use various washed-cell preparations of the biocatalyst to perform biotransformations. However, the presence of an active lactone hydrolase in the same catabolic pathway can result in low yields of lactone products unless a suitable inhibitor, such as tetraethylpyrophosphate, is added to the reaction mixture[8]. In bacteria such as camphor-grown *Pseudomonas putida*, which contains various induced BVMOs with conflicting selectivities, the use of whole-cell preparations is impractical[9]. Both problems can be eliminated by using purified enzyme preparations. One potential problem of using pure enzymes, namely the cost of using stoichiometric amounts of the relevant reduced nicotinamide nucleotide (Table 1), has been resolved by the successful use of appropriate linked-enzyme coenzyme-recycle systems (NADP/H + glucose dehydrogenase, NAD/H + formate dehydrogenase). The coenzyme recycling system for each BVMO has been developed as a continuously-operated process using enzyme membrane reactor-based technology[10,11]. By choosing the appropriate substrate and a suitable enzyme complement, it is possible to operate a closed-loop recycling procedure that eliminates the need for a sacrificial substrate (Fig. 1b).

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PII: S0167-7799(97)01004-4

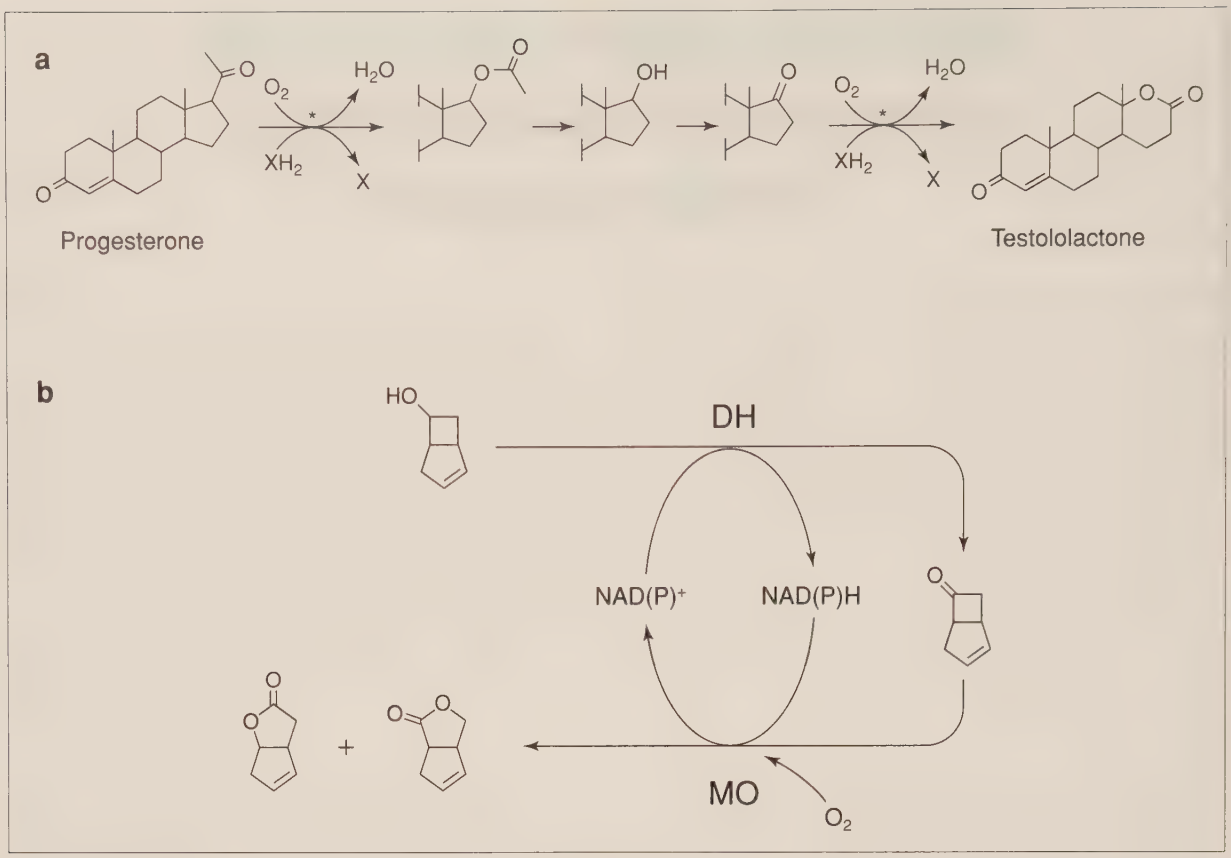

Figure 1

(**a**) Characterized pathway of progesterone metabolism in *Cylindrocarpon radicicola*[2], containing two examples (denoted by *) of Baeyer–Villiger monooxygenase catalysed reactions. (**b**) Intrasequential regeneration of nicotinamide nucleotide coenzymes using a coupled alcohol dehydrogenase (DH) and Baeyer–Villiger monooxygenase (MO)[12].

Structural features of BVMOs

The best characterized BVMO to date is cyclohexanone monooxygenase from *A. calcoaceticus*. The enzyme is monomeric with a molecular mass of 59 kDa: although a complete three-dimensional structure remains to be established (see below), a comprehensive series of studies by Trudgill's group has concluded that the enzyme has only one active site[13]. Some characteristics of CHMO and a number of other BVMOs that have been purified are compared in Table 1. Although the complete sequence is only available for CHMO (Ref. 14), partial N-terminal sequences have been obtained from a sufficient number of these flavoproteins (Box 1) to justify dividing them into two distinct types, known as Type 1 and Type 2 enzymes (G. Grogan, PhD Thesis, University of Exeter, 1995). Type 1 enzymes are FAD binding and NADPH-dependent, and both coenzymes plus the substrate all bind to the same polypeptide subunit: some of these enzymes are monomeric, whereas others have a homo-oligomeric quaternary structure. By contrast, Type 2 enzymes are FMN binding and NADH-dependent and consist of two distinct types of subunits, one of which binds the carbonyl substrate and the other the nicotinamide nucleotide, effectively serving as an NADH dehydrogenase.

A notable feature of all the Type 1 enzymes is the occurrence, as observed in a number of other FAD-binding enzymes, of the characteristic GxGxxG consensus nucleotide sequence located near to the N-terminus, which represents the binding site for the adenosine moiety of FAD (Ref. 15). Although it remains to be confirmed by further examples, it appears that a distinct feature of Type 1 BVMOs that distinguishes them from other FAD-dependent enzymes is the presence of an invariant aspartic acid residue immediately upstream from the FAD-binding site. The functional significance, if any, of this residue remains to be established. The N-terminal sequences of 2,5-diketocamphane 1,2-monooxygenase (2,5-DKCMO) and 3,6-diketocamphane 1,6-monooxygenase (3,6-DKCMO) [two Type 2 FMN-binding and NADH-dependent isozymes responsible for the degradation of (+)- and (–)-camphor, respectively, in *Pseudomonas putida*[16]] are clearly different from Type 1 enzymes but show considerable homology to each other as well as to various bacterial luciferases, which act as BVMOs in performing the FMN and NADH-dependent oxygenation of dodecanal to dodecanoic acid[17].

Enantiodivergent biotransformations including synthetic applications

The selectivities of some of the nucleophilic oxygenations performed by this group of enzymes are as unusual as they are impressive. In some cases, particular

Table 1. Characteristics of some microbial Baeyer–Villiger monooxygenases

Enzyme and source microorganism	Coenzyme	No. of proteins	Subunit composition	Molecular mass (kDa)	Mole coenzyme/ mole protein	pH optimum
Cyclohexanone monooxygenase *Acinetobacter calcoaceticus* NCIMB 9871	NADPH	1	Single polypeptide chain	59	1 FAD	9.0
Cyclohexanone monooxygenase *Nocardia globerula* CL1	NADPH	1	Single polypeptide chain	53	1 FAD	8.4
Cyclohexanone monooxygenase *Xanthobacter* sp.	NADPH	1	Single polypeptide chain	50	1 FMN	8.8
Cyclopentanone monooxygenase *Pseudomonas* sp. NCIMB 9872	NADPH	1	3–4 identical subunits	200	1 FAD	7.7
Tridecan-2-one monooxygenase *Pseudomonas cepacia*	NADPH	1	Two identical subunits	123	1 FAD	7.8–8.0
Diketocamphane monooxygenase *Pseudomonas putida* NCIMB 10007	NADH	2	(i) Two identical subunits (ii) One polypeptide chain	78 36	1 FMN	9.0
2-oxo-Δ^3-4,5,5-trimethylcyclopentenyl-acetyl-CoA monooxygenase *Pseudomonas putida* NCIMB 10007	NADPH	1	Two identical subunits	106	1 FAD	9.0
Steroid monooxygenase *Cylindrocarpon radicicola* ATCC 11011	NADPH	1	Two identical subunits	155	1 FAD	7.8
Oxocineole monooxygenase *Pseudomonas flava* UQM 1742	NADH	2	(i) Two identical subunits (ii) One polypeptide chain	70 30	1 FMN	8.5–9.0

Box 1. Sequence data for Types 1 and 2 Baeyer–Villiger monooxygenases

Type 1: FAD- and NADPH-dependent

Binding motif for adenosine moiety of FAD
N-term.....5-25 amino acids.....**G.**x.**G**.x.x.**G**.....

- Cyclopentanone monooxygenase from *Pseudomonas* sp. NCIMB 9872
[1]T T M T T M T T E Q L G M N N S V N D K L **D** V L L I **G** A **G**

- Cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871
[1]S Q K M D F **D** A I V I **G** G **G** F G **G** L

- Steroid monooxygenase from *Cylindrocarpon radicicola* ATCC 11011
[1]A E W A E E F **D** V L V V **G** A **G** A G **G**

- Cyclohexanone monooxygenase from *Rhodococcus* sp. WT1
[1]T A ? T I H ? V **D** A V V I **G** A **G** F G **G** I Y A V H K

- 2-Oxo-4,5,5-trimethylcyclopent-3-enylacetyl-CoA monooxygenase from *Pseudomonas putida* NCIMB 10007
[1]N R A K S P A L **D** A V V I **G** A **G** V T **G** I Y Q A F L I N Q A G M A V L

Type 2: FMN- and NADH-dependent

- 3,6-Diketocamphane monooxygenase from *Pseudomonas putida* NCIMB 10007
[1]A M E T G L I F H P Y M R P G R S A A Q T F D W G I K ? A

- 2,5-Diketocamphane monooxygenase from *Pseudomonas putida* NCIMB 10007
[1]M Q A G F F G T P Y D I P T R T A R Q M

- Luciferase (luxA) from *Vibrio harveyi* ATCC 33843
[1]M.K.F.G.N.F.L.L.T.Y.Q.P.P.E.L.S.Q.T.E.V.M.K.R.L.V.N.L.G

- Luciferase (luxA) from *Vibrio fischeri* ATCC 7744
[1]M K F G N I S F S Y Q P S G E

- Luciferase (luxB) from *Vibrio harveyi* ATCC 33843
[1]**M** K F G L F F L N F M F S K R S S

- Luciferase (luxB) from *Vibrio fischeri* ATCC 7744
[1]M.K.F.G.L.F.F.L.N.F.Q.K.D.G.I

[1]indicates the N-terminal amino acid residue as determined by sequence analysis.

biotransformations have been used to yield chiral synthons that have themselves been incorporated into successful chemoenzymatic syntheses of various potentially useful molecules. With the exception of the luciferases from *Vibrio fischeri* and *Photobacterium phophoreum*[18], and the as yet uncharacterized activity in *Cunninghamella echinulata*[19], all other BVMOs examined, including pure enzyme preparations of various Type 1 and Type 2 enzymes, perform an enantiodivergent biotransformation of the racemic ketone bicyclo[3.2.0]hept-2-en-6-one to yield two homochiral lactones. This indicates that the two ketone enantiomers have been discriminated absolutely on a regio- plus enantioselective basis (Fig. 2a). Such a spectacular outcome has been confirmed using the separate substrate enantiomers with a pure preparation of CHMO (Ref. 21). Equally remarkable is that the outcome from 2,5-DKCMO represents a mirror-image of the CHMO-catalysed biotransformation, thereby providing access to all four possible bicyclic [3.3.0] lactones in homochiral form (Fig. 2a). Interestingly, the outcome can be influenced significantly by the presence of chemical functionalities at either the 2 plus 3 or 7 plus 7′ positions[23], a valuable feature in developing active-site models of these enzymes (see below). These, and equivalent, biotransformations of various other unsubstituted bicyclic ketones containing a furan or pyran ring have been used to yield chiral synthons that can themselves be incorporated into chemoenzymatic syntheses of useful compounds as diverse as the pheromones multifidene and viridiene, the antibiotic sarkomycin A and the insect antifeedant clerodin (Fig. 2b). Currently, such biotransformations are undoubtedly the best way to achieve asymmetric Baeyer–Villiger oxidations, although recently some limited enantioselectivity has been achieved in equivalent chemical reactions catalysed by various expensive metal-based reagents[26,27].

Kinetic resolutions including synthetic applications

With other racemic substrates – including 2-bromo-3-hydroxy-substituted [3.2.0] ketones[23], various substituted [2.2.1] ketones[28] and 2′-substituted monocyclic ketones[8,29] – both CHMO and the two DKCMO isozymes perform a very different type of regio- plus enantioselective biotransformation; each case yielding by kinetic resolution a single homochiral lactone (Fig. 3a). Changing the nature and/or length of the 2′-side-chain of the monocyclic substrates can have a significant effect on both the outcome and yield of these biotransformations[8]. Biotransformations of this type have been exploited to yield key chiral synthons for chemoenzymatic syntheses. Useful targets produced in this way have included potent antiviral carbocyclic nucleosides[30], a key precursor for the synthesis of the powerful insect antifeedant azadirachtin[28], and (*S*)-5-hexadecanolide, the female sex pheromone of *Vespa orientalis*[31].

Another such application, the successful chemoenzymatic synthesis of the important coenzyme (*R*)-lipoic acid, illustrates a number of points about the use of these enzymes to perform useful biotransformations (Fig. 3b). Conventional retrosynthetic analysis identified a route to (*R*)-lipoic acid in which (*S*)-methyl 6,8-dihydroxyoctanoate, obtained indirectly from the appropriate regio- plus enantioselective BVMO-dependent biotransformation of racemic 2-(2′-acetoxyethyl)cyclohexanone, would achieve the desired objective[29]. A BVMO from *P. putida*, 2-oxo-4,5,5-trimethylcyclopent-3-enylacetyl-CoA monooxygenase, performed a kinetic resolution with the required regioselectivity but the wrong enantioselectivity. Hydrolysis of the resultant (*R*)-(*E*)-lactone gave, the (*R*)-diol, which could be converted into the required (*S*)-diol by performing additional chemical steps. Further investigation[32] revealed that cyclopentanone monooxygenase from cyclopentanol-grown *Pseudomonas* sp. NCIMB 9872, an enzyme previously noted for its poor enantioselectivity with various other ketone substrates, was able to yield the (*S*)-(*E*)-lactone, thereby completing the original planned synthetic route.

Biotransformation of *meso* ketones

Some highly selective biotransformations have also been recorded with prochiral ketone substrates such as 4-methylcyclohexanone. This and other *meso* compounds, including various bi- and tricyclic ketones, were asymmetrized to homochiral lactone products (e.e. > 98%) by CHMO (Ref. 5). The ability of other BVMOs, including those from *P. putida*, to perform equivalent asymmetrizations has been confirmed by a recent study of various substituted cyclobutanones, which, interestingly, identified some substrates that were biotransformed by both CHMO and 2,5-DKCMO with the same enantioselectivity[33]. Some of these biotransformations have been exploited to yield chiral synthons with potential value for the chemoenzymatic synthesis of many useful compounds, including the antibiotic ionomycin[5] and various lignan derivatives with interesting antileukaemic activities[33].

Active site modelling of BVMOs

To understand the exquisite stereoselectivity of BVMOs, and to make predictions of the likely products from untested substrates of commercial interest, it is necessary to know the three-dimensional structure of potentially useful biocatalysts such as CHMO and the DKCMO isozymes. To date, no such structures have been established. Full amino acid sequences are known only for CHMO from *A. calcoaceticus*, although there are partial sequence data for various other enzymes (Box 1). Good progress is being made on a joint international programme to coordinate investigation into the three-dimensional structure of CHMO and the DKCMO isozymes; the parties involved are the universities of Exeter, UK (A. Willetts); London, UK (J. Ward); Edinburgh, UK (S. Chapman, G. Reid); Milan, Italy (G. Ottolina) and Brock, Canada (H. Holland). In the absence of any full structures, a

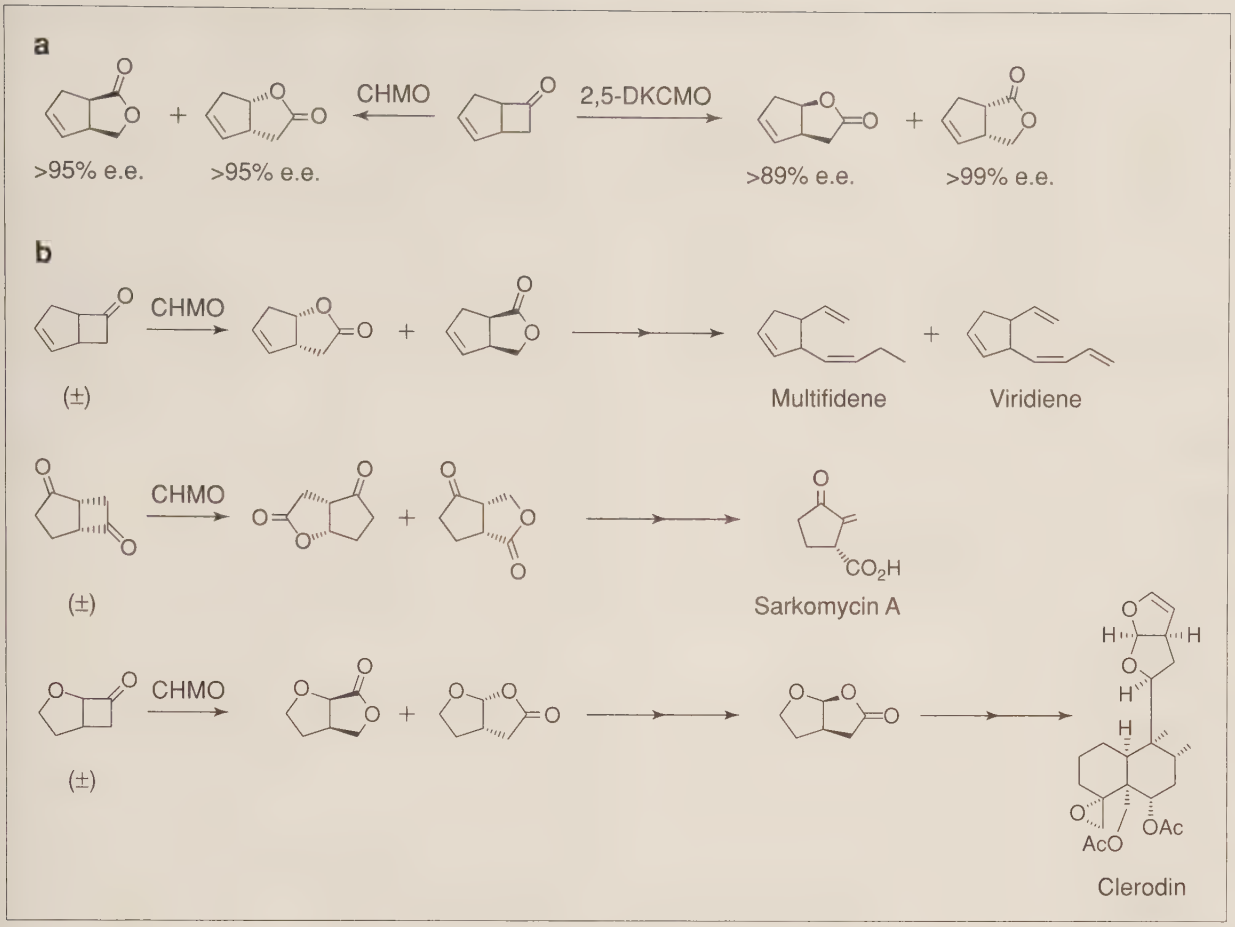

Figure 2

(**a**) Enantiodivergent biotransformation of bicyclo[3.2.0]hept-2-en-6-one by cyclohexanone monooxygenase (CHMO)[20] and 2,5-diketocamphane 1,2-monooxygenase[22]. (**b**) Chemoenzymatic syntheses of various useful target molecules (multifidene and viridiene[23], sarkomycin A[24] and clerodin[25]) involving the biotransformation by (CHMO) of bicyclo[3.2.0]-type ketones.

number of attempts have been made to develop models that explain the outcomes of biotransformations performed by these enzymes. Three very different approaches have been used but there is significant comparability between some outcomes in the case of CHMO from NCIMB 9871, the most widely modelled enzyme to date.

'Cubic space' models of BVMOs

One approach has been to exploit some form of 'cubic space' approach[34] to develop an active site model. It has been assumed that the outcome of events in the active site will be determined by a combination of the stereochemical and the stereoelectronic effects originally devised for the hypothetical enzyme mechanism[35] and the equivalent chemical reaction[36]. The model of CHMO proposed by Furstoss (Fig. 4a) has now been developed through several iterations to take account of the outcomes of an increasing number of ketone biotransformations[1]. It is based on a regular cubic space containing 'forbidden zones' that represent areas of steric hindrance. The confines of the model are established by testing the theoretical positions that may be adopted by the transition state intermediates of any particular biotransformation, and matching these with the actual outcome.

A similar approach has produced the Milan–Brock model of the active site of CHMO, which is probably the most realistic to date and the only one to explain the outcomes of both sulfoxidation[37,38] and lactonization[39] biotransformations (Fig. 4b). In this case a molecular modelling program (Hyperchem) was used to minimize the total energy of each biotransformation product. The processed structures were then superimposed, either at the S atom along the S=O bond or the O atom along the C–O peroxide bond, to define various binding pockets that emerge from inserting the minimized structures inside a 'cage' initially composed of several cubes of 1.54 Å edge. The model can even explain the recorded outcomes with cyclic thiolactones, which are known to be suicide inhibitors of CHMO (Ref. 40).

Mechanism-based models of BVMOs

The other models that have been produced are more mechanism-based. Taschner proposed such a model for CHMO by using a very different approach[5]. He argued that the active site of this flavoprotein is likely to be similar to that of human and *Escherichia coli* glutathione reductase, two other FAD-binding proteins for which the three-dimensional structure is already known. The N-terminal sequence of all three proteins

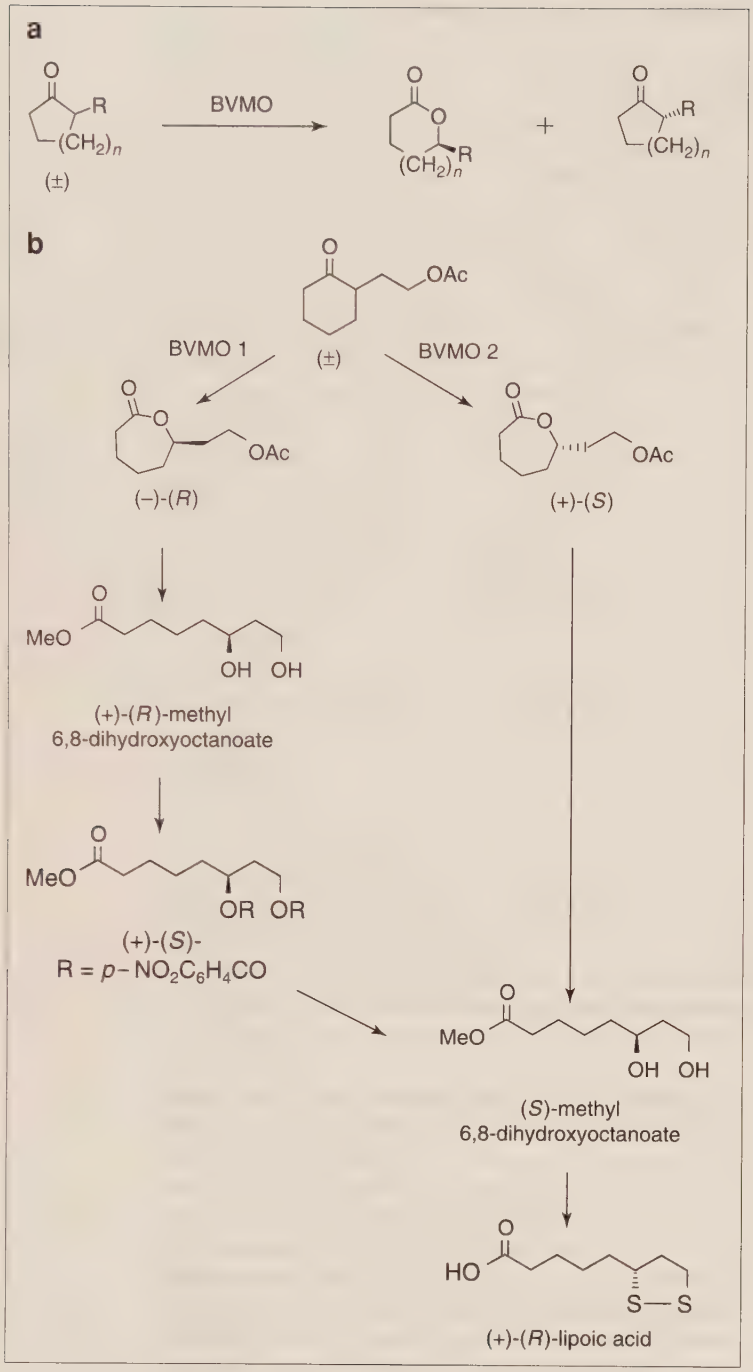

Figure 3

(**a**) Kinetic resolution of 2′-substituted monocyclic ketones by Baeyer–Villiger monooxygenases (BVMO). (**b**) Chemoenzymatic synthesis of (*R*)-(+)-lipoic acid by either 2-oxo-4,5,5-trimethylcyclopent-3-enylacetyl-CoA monooxygenase (BVMO 1; Ref. 29) or cyclopentanone monooxygenase (BVMO 2; Ref. 32).

is highly conserved and includes a similar BAB–domain incorporating the same GxGxxG FAD(=adenosine)-binding motif[15]. Taking this as a starting point, and then applying the same stereochemical and stereoelectronic considerations used to formulate the 'cubic space' models, Taschner developed a model that successfully explained the observed outcomes from various *meso* ketones (Fig. 4c). A key feature is that the hydroperoxide of the oxygenated FAD intermediate was assumed to be attached to the *re*-face of the isoalloxane ring of the flavin cofactor, and that the ketone substrate approaches the hydroperoxide from the direction of the dimethylbenzene moiety. This mechanism-based model is compatible with those developed by the alternative 'cubic space' approach.

Kelly has proposed a second mechanism-based model (Fig. 4d), developed initially from the outcome of the biotransformation of a single, unsubstituted tricyclic ketone by CHMO (Ref. 41). A basic difference between this model and the others is that it is proposed that enantioselectivity is controlled exclusively by stereoelectronic considerations, being a function of the diastereofacial selectivity of the enzyme for the substrate. The likely influence of stereochemical considerations imposed by the interaction of the ketone substrate and the confines of the active site was not considered, despite the existence of data that clearly demonstrate the dramatically different outcomes of the biotransformation of unsubstituted versus substituted bicyclic [3.2.0] ketones (*vide supra*). In another substantial departure from Taschner's model, it was assumed that, in the transition state intermediate formed in the active site of CHMO, the hydroperoxide is attached to the *si*-face of the isoalloxane ring of FAD, and that the ketone substrate approaches the hydroperoxide from the direction of the diazine moiety. The resultant transition state intermediate was described as having an '*S*-migration configuration' when the Cahn–Ingold–Prelog rules were applied. It was proposed that the enantioselectivity of the resultant biotransformation was dictated by this '*S*-migration configuration'. When extended to the outcomes of equivalent biotransformations, both DKCMO isozymes were defined as establishing '*R*-migration configuration' transition state intermediates, thereby yielding a different lactone product. Kelly's model has its merits as an explanation for observed outcomes from the tricyclic ketone substrate, but fails to explain both outcomes of sulfoxidation by these enzymes[42] and why CHMO and the DKCMO isozymes biotransform some substituted cyclobutanones with identical enantioselectivity[33].

Concluding remarks

A comprehensive explanation of some of the bizarre selectivities observed with this interesting group of enzymes must await the characterization of the relevant three-dimensional structures. However, the values of these enzymes in asymmetric synthesis, enabling convenient access to chiral lactones and sulfoxides, has been clearly demonstrated. Further applications of this emerging field of biotransformations will undoubtedly follow. Although no large-scale syntheses have been commissioned to date, a number of the enabling technologies, including the relevant coenzyme recycling methodologies, have been successfully developed already: further issues, including the important matter of ensuring adequate oxygen availability to optimize the biotransformations, are currently being addressed by an on-going research programme involving the UK universities of Exeter and Liverpool, and University College London.

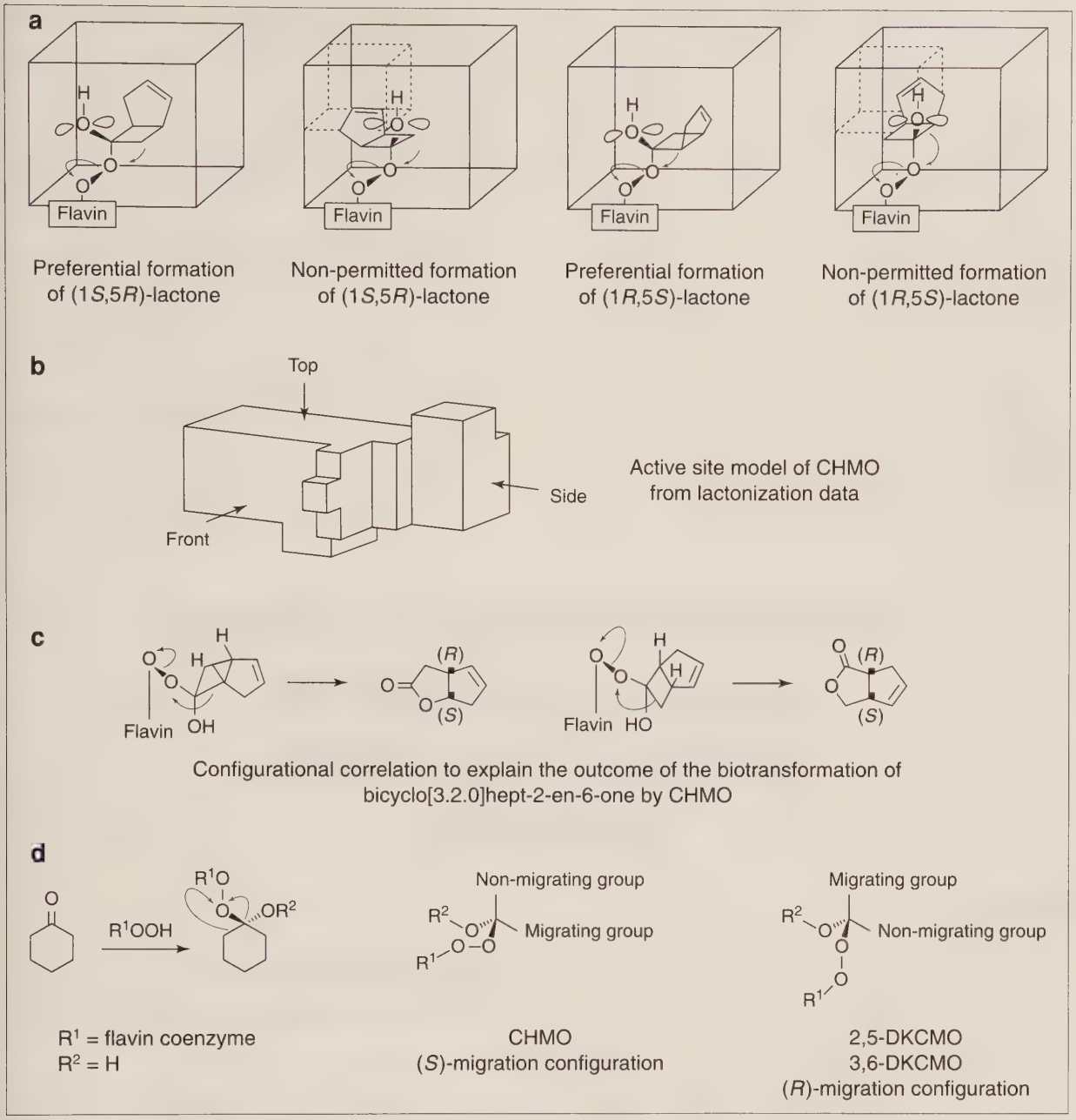

Figure 4

Active site models of cyclohexanone monooxygenase (CHMO). (**a**) 'Cubic space' model proposed by Furstoss *et al.*[1]: 'the forbidden zone' is outlined by the dotted lines. (**b**) 'Cubic space' model proposed by Ottolina *et al.*[37–39] (**c**) Mechanism-based model proposed by Taschner *et al.*[5] (**d**) Mechanism-based model of Kelly[41,42].

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Designing and optimizing library selection strategies for generating high-affinity antibodies

Hennie R. Hoogenboom

Since its invention at the beginning of the 1990s, antibody phage display has revolutionized the generation of monoclonal antibodies and their engineering. It is now possible to create antibodies binding to any chosen target antigen without the use of laboratory animals or hybridomas, in a system that completely by-passes the immune system. Making antibodies from single-pot phage libraries, and improving their affinity up to the picomolar range if necessary, has never appeared easier. In this review, a variety of phage library-based strategies for the isolation of high-affinity antibodies are presented.

The biotechnological generation of high-affinity monoclonal antibodies has traditionally involved the production of hybridomas from spleen cells of immunized animals. Now the use of phage antibodies offers a new route for the generation of antibodies, including antibodies of human origin, which cannot be easily isolated by conventional hybridoma technology. Over the last decade, protein engineering methods to generate more human-like antibodies have been developed[1] (chimerization and humanization of rodent antibodies) and, more recently, transgenic mice expressing human antibodies were proposed (reviewed in Ref. 2). In all cases, antibodies are created or engineered with their specificity and affinity shaped by the immune system. With phage display, antibodies can be made completely *in vitro*, by-passing the immune system and the immunization procedure, and allowing *in*

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PII: S0167-7799(96)10070-6

vitro tailoring of the affinity and specificity of the antibody. As for other methods, the first commercially significant application of phage antibodies is the generation of fully human monoclonal antibodies for medical use. In fact, the use of phage display is set to gradually replace hybridoma technology in all of its present applications, in both research and in the diagnostic industry.

Antibody phage display[3] is accomplished by fusing the coding sequence of the antibody variable (V) regions to the amino terminus of the phage minor coat protein pIII. Expression of the fusion product and its subsequent incorporation into the mature phage coat results in the antibody being presented on the phage surface, while its genetic material resides within the phage particle. This linkage between antibody genotype and phenotype allows the enrichment of antigen-specific phage antibodies, using immobilized or labelled antigen. Phage that display a relevant antibody will be retained on a surface coated with antigen, while non-adherent phages will be washed away. Bound phages can be recovered from the surface, re-infected into bacteria and re-grown for further enrichment and, eventually, for binding analysis. The success of antibody phage display hinges on the combination of this display and enrichment method, with the creation of large combinatorial repertoires of phage antibodies. Each of these antibodies has a different antigen combining site, generated by a PCR-based amplification of antibody V-genes (reviewed in Ref. 4). An antigen-driven enrichment procedure can be used to isolate even the rarest phage antibody. The complete procedure is illustrated in Fig. 1. This basic technique has been applied to construct very large and highly diverse combinatorial repertoires of antibody fragments, from which antigen-specific antibodies to any chosen antigen can be selected[5]. This review will focus on the applications, advantages and disadvantages of these large repertoires.

Choosing the right antibody library

The choice of which type of phage antibody repertoire to use depends very much on the application and the required affinity of the antibody. Ideally, antibodies to any chosen antigen are selected from universal, antigen-unbiased libraries. Two types of such 'single-pot' libraries have been described, namely the naive and synthetic antibody libraries. Indeed, it has been demonstrated that these can be used as a source of antibodies against any chosen antigen. The alternative is to develop and use antigen-biased phage antibody repertoires, using V-genes from immune sources. Figure 2 shows a decision tree to guide the choice of the repertoire (points 1–3), and the selection strategy (4–6), depending on a number of parameters. Figure 3 shows a comparison of the performance of different repertoires, which is discussed in detail below.

Antibodies from naive V-gene libraries

The primary immune response involves a large array of IgM antibodies that recognize a variety of antigens. This array can be cloned as a 'naive' repertoire of rearranged genes, by harvesting the V-genes from the IgM mRNA of B cells of unimmunized human donors[6], isolated from peripheral blood lymphocytes (PBLs), bone marrow, spleen cells or from animal sources[7]. The V-genes are amplified from the cDNA using oligonucleotides binding to the Framework (FR) 1 and 4 encoding regions of the V-gene, and heavy and light chains assembled randomly to encode single-chain Fv (scFv) or Fab antibodies. These cassettes are cloned directly into an appropriate phage or phagemid vector for expression and display. The first of such 'single-pot' repertoires[6] was made from the PBLs of two healthy human volunteers and contained 3×10^7 clones, from which antibodies to over 25 different antigens were isolated. These included antibodies to self antigens and cell-surface molecules[6,8]. The average affinity of these antibodies was in the 10^6–$10^7\,M^{-1}$ range, which is similar to the affinity of antibodies seen in the naive primary immune response. Recently, much larger scFv repertoires have been made by 'brute force' cloning[9], with 10^9–10^{10} independent antibody clones, and from these libraries higher-affinity antibodies (K_a around $10^9\,M^{-1}$) have been selected. Even larger-sized naive Fab repertoires may be made by using the *in vivo* recombination system based on the *Cre/loxP* recombination system[5]. In this method, individually cloned light and heavy chain repertoires are recombined using appropriately positioned *loxP* recombination sites by P1 bacteriophage *Cre* recombinase, yielding Fab repertoires that in size are limited by the amount of bacteria grown, typically 10^{10}–10^{13} members.

The major advantages of using very large naive single-pot repertoires are: (1) one library can be used for all antigens; (2) human antibodies can be isolated; (3) antibodies to self, non-immunogenic or toxic substances can be generated; (4) antibody generation takes 2–4 rounds of selection (approx. two weeks); and (5) when very large repertoires are available, high-affinity antibodies are isolated directly.

The disadvantages are: (1) the lower affinity of the antibodies when smaller-sized repertoires are used; (2) the time and effort it takes to construct these repertoires, in particular when using the *Cre/loxP* recombination system[5]; and (3) the largely unknown and uncontrollable contents of these naive libraries. In particular, the potentially limited diversity present in the IgM repertoire, its bias due to unequal expression of the different V-gene families, and the unknown immune history of the B cell donor, influence the contents and quality of the library. Therefore, an alternative method, based on the controlled addition of defined and chosen V-gene building blocks to antibody repertoires, has been developed: the synthetic antibody library.

Antibodies from synthetic V-gene libraries

The wealth of data on the *in vivo* generation of antibodies, via a complex process of DNA rearrangement, and the many antibody structures that have been

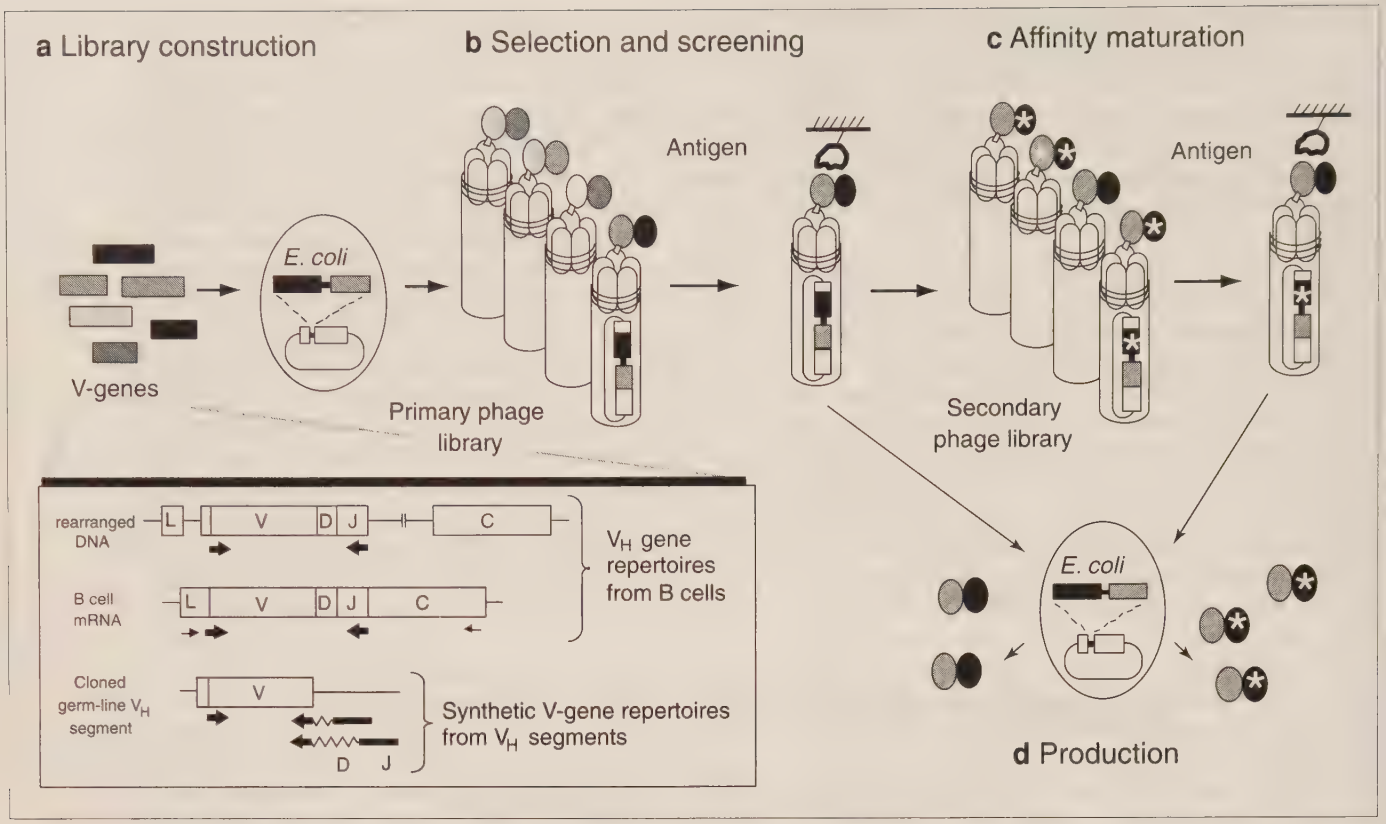


Figure 1

Overview of the phage display method. (**a**) Library construction: V-genes (grey and black boxes) are cloned in phage(mid) vectors for display on phage. (**b**) From these primary libraries, antigen-specific antibodies are enriched using affinity selection. (**c**) If necessary, the selected antibodies are affinity matured, by diversifying their sequence, yielding a secondary library from which the highest affinity clones are enriched. (**d**) Finally, soluble antibody fragments may be produced by expression in bacteria. The inset highlights the location of oligonucleotides for cloning of V_H segments, used for amplifying the rearranged genes from DNA or mRNA (for naive or immune libraries), or to provide an artificial CDR3 (as an example for synthetic libraries). L, leader; V, variable; D, diversity; J, joining; C, constant domain segments. Modified from Ref. 4.

solved have shown clearly that most of an antibody's binding strength resides in the six complementarity determining regions (CDRs) that shape the antigen combining site. This knowledge has paved the way for the creation of antibodies entirely outside their natural host. To construct a synthetic antibody library, V-genes are assembled by introducing a predetermined level of randomization of CDRs (or possibly also bordering FR-regions) into germ-line V-gene segments[10], or rearranged V-genes[11]. One of the main advantages over naive repertoires is that in such synthetic repertoires the contents, local variability and overall diversity of the library may be controlled and defined.

The areas of synthetic diversity are mostly chosen to correspond to areas of greatest natural sequence diversity of the primary antibody repertoire. From structural studies it has become apparent that five of the six CDR regions (all but the CDR3 of the heavy chain) have limited structural variation, and frequently follow a certain canonical fold[12]. The greatest structural diversity is found in the loop most central to the antigen combining site, the CDR3 of the heavy chain. This loop is highly diverse in composition and in length owing to the *in vivo* recombination procedure.

The antibody CDR regions may be partially or completely randomized using oligonucleotide-directed mutagenesis or PCR-based methods. Several synthetic or semi-synthetic repertoires have been made in which, in particular, the heavy-chain CDR3 loop was targeted. In the first synthetic antibody library, constructed in Cambridge, UK, a set of 49 human V_H-segments was assembled via PCR with a short CDR3 region (encoding either five or eight amino acids) and a J-region, and cloned for display as a scFv with a human lambda light-chain[10]. From this repertoire, many antibodies to haptens, and one against a protein antigen, were isolated, delivering the first proof of concept for this synthetic antibody approach. Subsequently, the CDR3 regions were enlarged to match better the natural length diversity of this loop[13], and eventually 47 light-chain segments with partially randomized CDR3 were added[5]. In the final repertoire a set of nearly 100 cloned human V-gene segments was used, covering over 95% of the segments used in the natural human repertoire, and therefore resembling the human primary antibody library in its diversity[5]. In other synthetic antibody repertoires, one rearranged V-gene pair was provided with a one-length randomized CDR3 region in the heavy chain[11], or all three CDR-loops in one antibody V-gene were diversified[14]. Many different synthetic library concepts appear to work in creating antibodies of intermediate affinity

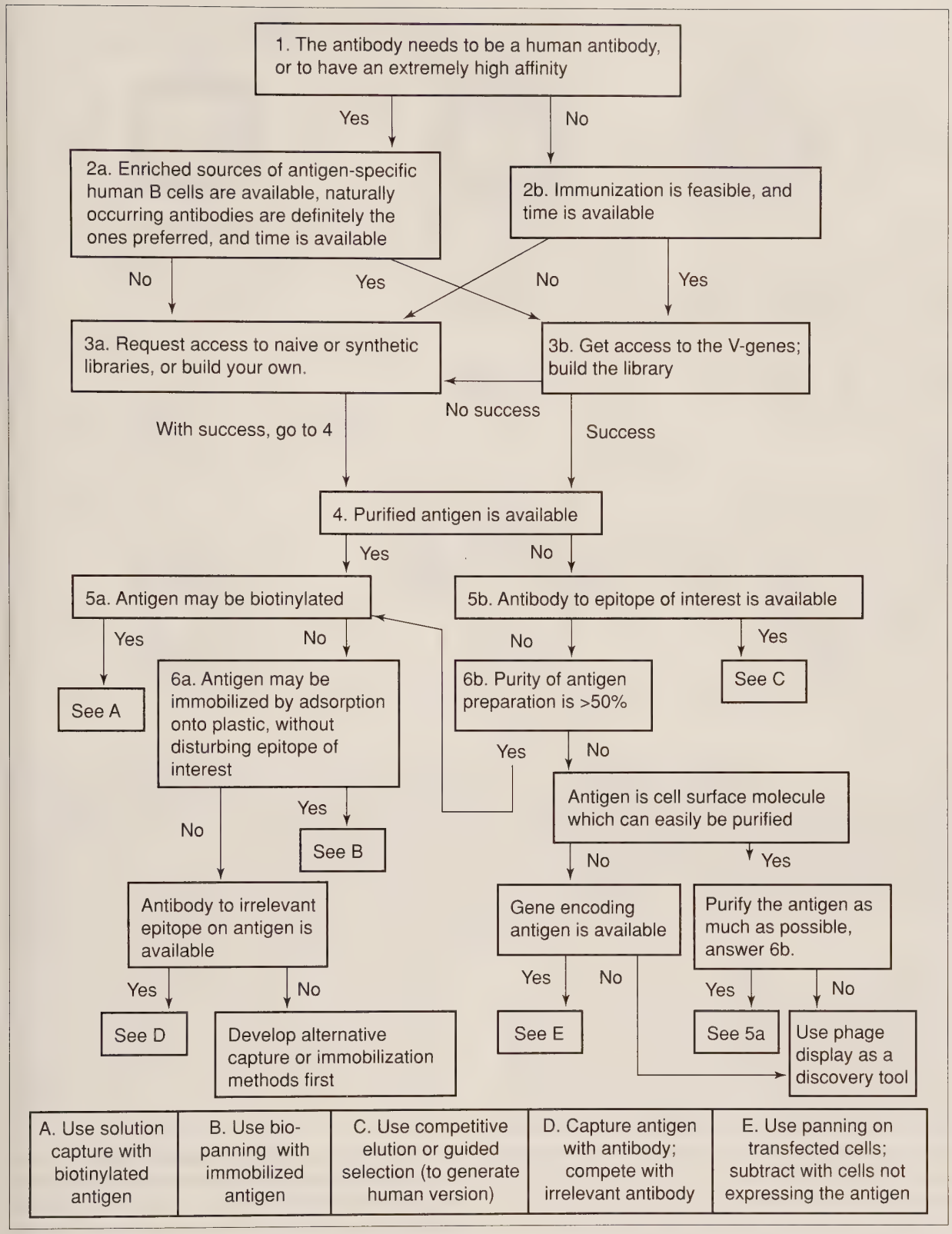
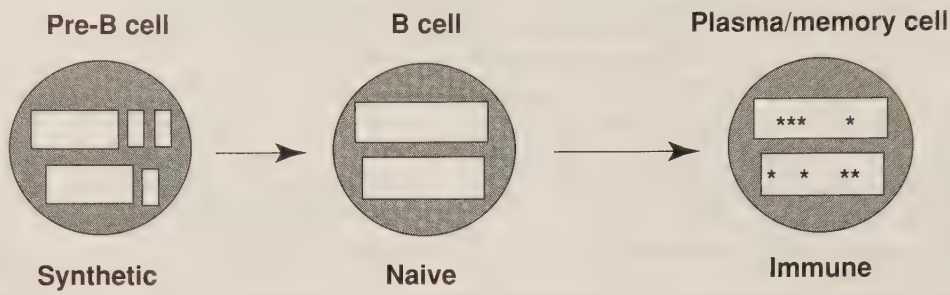


Figure 2

Flow chart to decide which type of antibody library to make or use (1–3), and which selection method to apply (4–5).

(10^6–10^8 M^{-1}). However, in all cases the cloning of a sufficient number of variants will become limiting. For example, when using mutagenic oligonucleotides to randomize six codons (with, at each triplet, the first two base positions A, G, C or T, and the last G or C) one billion combinations arise. Limitations in transformation efficiency limit the size of the antibody library that can be made in practice to 10^{9-10} variants and, therefore, the total accessible diversity. A combination of the dual antibody cloning strategy[15] and a smart cloning system, based on *in vivo Cre/loxP* site-specific recombination, allows the construction of very



	Synthetic	Naive	Immune
V-gene source	Unrearranged V-gene segments	Rearranged V-genes from IgM pool	Rearranged V-genes from IgG pool
Contents	Controlled	Uncontrolled	Uncontrolled
Repertoire construction	Once (Single-pot)		New repertoire for every antigen
Affinity of antibodies	Depending on library size: μM from standard size repertoire (10^7) nM from very large repertoire (10^{10})		Biased for high affinity (nM; if antigen is immunogenic)
Specificity	Any	Originally biased against self	Immunodominant epitopes, biased against self

Figure 3
Comparison between synthetic, naive and immune libraries.

large repertoires[5], with 10^{10-13} members. By using larger repertoires, the chances of finding a higher-affinity antibody are increased. Indeed, from a repertoire with 6×10^{10} clones, Griffiths and colleagues isolated synthetic Fab fragments with high affinity (K_a 10^8–10^9 M^{-1}).

Artificial versus natural

Synthetic antibody repertoires containing genuine human germ-line sequences[5,10,13] deliver antibodies that may be considered more 'natural' than those from naive IgM-mRNA-derived repertoires, which are frequently found to be somatically mutated[6,8]. On the issue of immunogenicity, the randomized CDR3 may appear synthetic and thus foreign; however, the choice for relatively short (4–12 residue) CDR3 loops favours the lowest possible immunogenicity, without excluding fully (as with 'natural' human antibodies) anti-idiotype responses.

One of the main advantages over naive repertoires is that in synthetic repertoires the contents, local variability and overall diversity of the library may be controlled and defined. For example, in addition to the CDR3, 'primary' diversity may also be introduced in the subset of CDR1-2 residues that are hypervariable owing to the natural somatic hypermutation mechanism[38], rather than in all CDR residues[14]. A particular example is the provision of the CDR3 regions, which may be performed for germ-line segments individually, allowing choice of particular segments, and avoiding the frequent cross-overs noted when PCR-amplifying V-genes in batches[23]. After selection, CDR or 'Vernier' zone FR-regions may be varied at will, in parallel or sequentially. This may facilitate systematic manipulation by, for example, using standardized methods to perform affinity maturation by sequential CDR-diversification (see also below). Although the overall impression now may be that slightly better antibodies are generated from naive[9] than from synthetic repertoires[5], the advantages in using synthetic antibody libraries will eventually reduce primary naive libraries to useful but transient sources of V-genes.

Antibodies from immune V-gene libraries

For immune libraries, V-genes are derived from the IgG mRNA of B cells of an immune source, e.g. immunized animals or in some instances human immune B cells. An immune phage antibody library repertoire will be enriched in antigen-specific antibodies, some of which will also have been affinity matured by the immune system[16,17]. This method gives access to more, and sometimes better, antibodies than when working with hybridomas. For example, from an immune murine phage antibody library, Chester and colleagues identified an anti-CEA antibody with an affinity substantially higher than ever obtained with

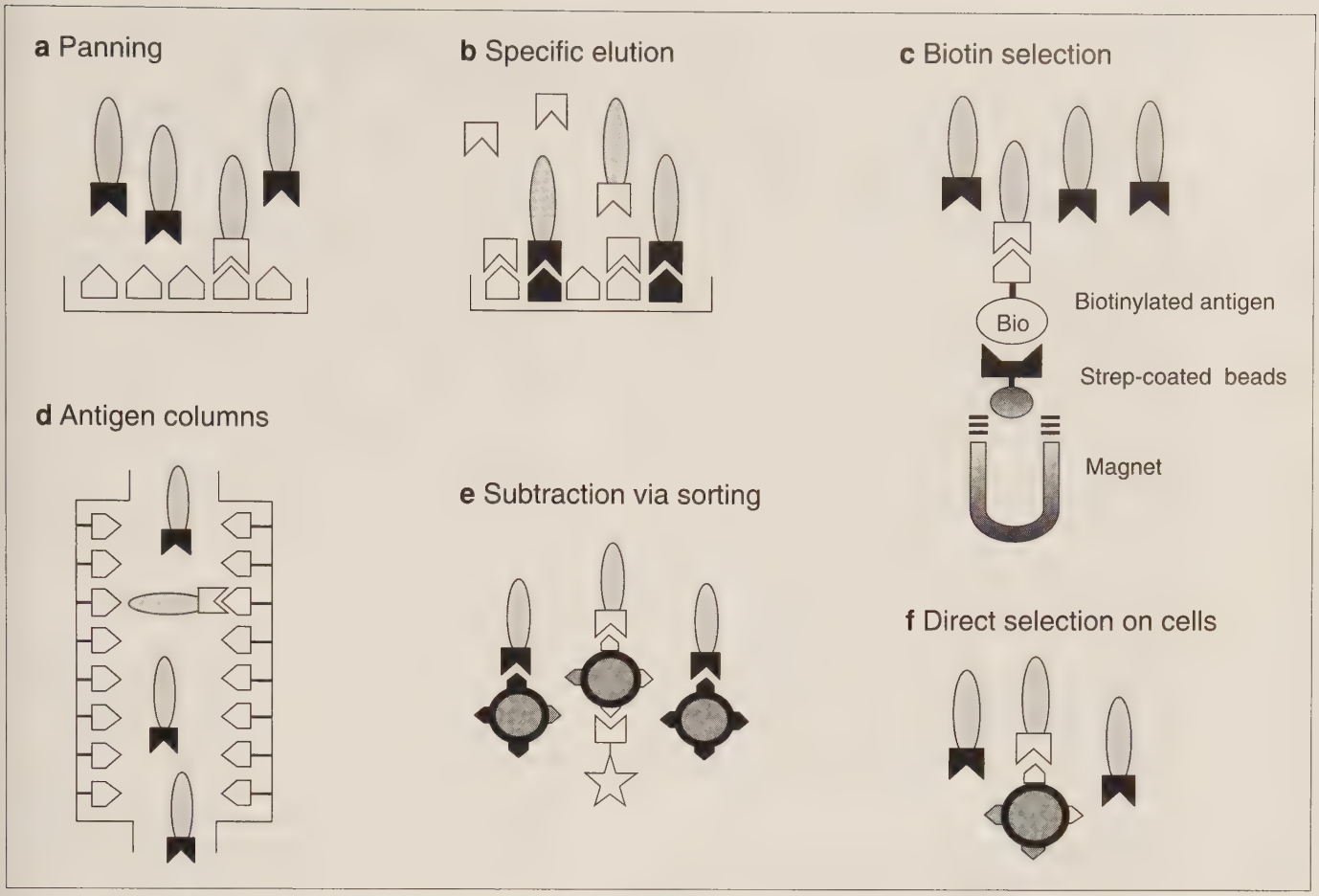


Figure 4

Most frequently used selection methods. Phages displaying antigen-specific antibodies (white) are usually separated from non-binding phages (black) using the following procedures. Antigen may be adsorbed onto Petri dishes or plastic tubes for panning (**a**), or immobilized on Sepharose and loaded onto columns for affinity selection (**d**). The antigen concentration used during selection may be monitored carefully by using antigen labelled with biotin. After incubation of phages with the biotinylated antigen, phages bound to the antigen–biotin complex are retrieved with streptavidin-coated paramagnetic beads and a magnet (**c**). When using impure antigen preparations or targeting a specific epitope, the antigen itself or other reagents that bind the antigen may be used to elute-off epitope-specific antibodies or cover irrelevant epitopes (**b**). For cell surface antigens, phages may be directly selected on cells (**f**), or antigen-displaying cells may be sorted by virtue of a marker unique for these cells, using fluorescence or magnetic activated cell sorting (FACS or MACS, respectively) (**e**). Combinations and alternations between these methods may be made. After washing, the bound phages can be eluted non-specifically, with high or low pH buffers, by changing the ionic strength or by direct incubation with bacteria. A protease recognition site may be encoded between the antibody fragment and the pIII protein, allowing specific protease cleavage (for references see Ref. 4).

the traditional technology[18]. Disadvantages of this route include the time required to immunize animals, the unpredictability of the immune response to the antigen of interest, the lack of immune response to some (for self antigens, or toxic molecules) and the restriction in generating human antibodies. In addition, a major drawback of this method is that for every new antigen a new phage antibody library has to be created, which adds 1–3 months to the procedure. Commercially available kits, such as RPAS from Pharmacia Biotech for the creation of scFv repertoires from mouse or rat spleen cells, will make access to this route easier for laboratories starting this work. However, the creation of such repertoires remains a technically non-trivial procedure and, therefore, for most applications only medium-sized repertoires will be made. The use of very large naive or synthetic libraries, when accessible, may well yield antibodies with similar affinities much faster. For example, the affinities of the best antibodies directed to the hapten 2-phenyloxazol-5-one, selected from an immune repertoire[16], were of the same range as those isolated from a very large synthetic repertoire[5]. Thus, the availability of pre-made large collections of non-immune repertoires has superseded the use of immune repertoires. It should be carefully considered if immunization and the creation of animal-derived antibody libraries is required, and also whether such experiments can be justified from an ethical point of view.

Immune repertoires for specialized users

There may be a few occasions where this route will still be useful, e.g. for the analysis of the humoral immune response in man. Naturally occurring high-affinity human antibodies, for example, present after viral infection or in patients with autoimmune diseases or cancer, may be cloned by phage display, with caution. The scrambling of V_H and V_L genes in the process of

the combinatorial library construction makes it difficult to discriminate 'original' V_H–V_L combinations from *de novo* formed pairs. The likelihood of retrieving original pairs is very low and, if necessary, 'in-cell PCR', in which V_H and V_L genes are assembled within the cell[19], may be used (although this method has only been demonstrated for mixed hybridomas). It has also been observed that the fine specificity may be shifted by mispairing the V-domains. Antibodies from immune random combinatorial libraries may identify the antigen or epitope(s) involved in the humoral immune response, and it may not always be strictly necessary to obtain original pairs.

In a second application of immune libraries, the immune response itself is used to remove irrelevant antibodies, thus improving the quality of the V-genes before cloning. Animals can be made tolerant for certain irrelevant antigens, after which the relevant antigen as a mixture with irrelevant antigens is used for immunization. This combination of *in vivo* and *in vitro* depletion and enrichment (tolerization and immunization *in vivo*, and depletion of phage antibodies before selection on antigen *in vitro*) should be extremely powerful, as exemplified by the isolation of human antitumour cell antibodies from phage repertoires of cancer patients immunized with autologous tumour cells[20].

Designing selection procedures

Phage antibody libraries are enriched for antigen-binding clones by subjecting the phages to repetitive rounds of selection that include binding, washing and elution steps, reinfection into bacteria and growth to re-express the antibody on the phage surface. Figure 4 shows the most frequently used *in vitro* selection methods, which are routinely used when purified antigen is available; Fig. 2 addresses which of the many methods should be used. Selections on impure antigens are significantly more difficult, owing to the problem of enriching phage antibodies specific for non-relevant antigens. Depletion and/or subtraction methods, cell sorting or selection by alternating between different sources of antigen may be used. Furthermore, the use of immune libraries with a higher frequency of specific antibodies may prove beneficial[20]. Using these fine-tuned methods, antibodies have been made to antigens previously thought of as difficult, such as to MHC–peptide complexes[21] and human blood group antigens[22].

Selection from phage antibody libraries provides a new tool for the isolation of novel self antigens, such as tumour- or disease-associated antigens. The *de novo* combined V-domain pairs in both naive and synthetic antibody libraries (but particularly the latter) are not shaped by the constraints of the immune system, and avoid library bias by *in vivo* tolerance mechanisms. Therefore, antibodies to unique self-epitopes can be isolated, provided powerful cell panning, sorting or subtraction methods are available. To date, this application has been used for probing lymphocyte[23] and tumour cell surfaces[20], yielding a number of promising but as yet uncloned cell-type specific antigens. The lack or reactivity of the selected phage antibodies in immunostaining[20], their low affinity[23], or the low abundance of antigen complicates their full characterization. The isolation of 'specific' binders *per se* does not prove that these subtractive selection methods function; by direct panning on a live colorectal carcinoma cell line we recently generated a large panel of anti-tumour scFv antibodies (Zijlema *et al.*, unpublished). The exquisite epithelial cell origin of the antigen was determined by positive immunohistochemistry on colon tumours derived from cancer patients. Systematic studies to compare the various enrichment methods are required to understand and appropriately design the selection of phage antibodies on cell surfaces. Lastly, *in vivo* selections, in which the phage repertoires are injected directly into animals, may reveal phage antibodies specific for certain organ-specific antigens, as demonstrated for peptide-phages[24].

Designing screening assays

After the selection procedure, a fast and robust screening assay for binding is applied, ranging from a simple ELISA with coated antigen, bioassays for screening for direct neutralization upon binding, to whole cell ELISAs, FACS or immunocyto- or histochemistry. Phagemid vectors that serve a dual purpose, mediating the monovalent display of scFv or Fab fragments for selection, and allowing production of soluble antibody fragments tagged with signals for purification and detection, are widely used[15]. If an alternative antibody format (e.g. Fab, whole antibody) is required for the screening assay, fast V-gene recloning methods should be used[25]. Alternatively, the direct display and selection of other formats of antibodies such as bispecific antibodies[26] may be envisaged.

Human antibodies via guided selection

Even when no antigen is available or the antigen is not known, it is possible to generate antigen-specific human antibodies. We have recently applied the technique of 'guided selection'[27] to derive a human anti-CD30 antibody, without having antigen available (Klimka *et al.*, unpublished). In this experiment, selections were carried out by panning a Hodgkin's cell line carrying the CD30 antigen on its surface, avoiding cloning, expression and purification of the antigen. Guided selection has been applied previously to generate anti-hapten and several anti-protein antibodies using standard phage selection on purified antigen. In this chain-shuffling technique, a murine antibody serves to guide the selection of the human V-domains onto the antigen[27]. In the first step, one of the murine V-domains is replaced with a repertoire of human V-domains, the rodent–human chimaeras displayed on phage and antigen-binding pairs enriched from the library. In a second step, the remaining murine sequence is replaced, resulting in a complete human equivalent of the original antibody. The selected human CDR-regions may interact with the same epitope in a very different manner from the original antibody.

yielding similar[27] or worse affinities[28], but always antibodies targeting the same region on the antigen.

Improving affinities

By selection from very large repertoires (with $>10^{10}$ antibody variants), antibodies with (sub)nanomolar affinity may be obtained directly[5,9], making affinity maturation necessary in some specialized cases only. The affinity of antibodies selected from the more frequently used medium-sized naive, synthetic or immune phage libraries, however, is typically sufficient for use as research reagents, but often rather suboptimal for therapeutic applications, i.e. tumour targeting and virus neutralization. Affinity maturation may be bypassed by the construction of multivalent molecules, as reviewed in Ref. 29; however, there will be occasions in which *in vitro* affinity maturation of the selected antibodies is required. *In vivo* antibodies are affinity matured in a stepwise fashion, by gradually incorporating mutations that each cause a small incremental improvement of the affinity. Mutations may affect affinity indirectly by influencing the positioning of side chains contacting the antigen, by providing new contact residues (particularly when they are located in or near the centre of the antigen combining site) or by replacing 'repulsive' or low-affinity contact residues with contact residues with more favourable energetics. The *in vitro* affinity maturation process essentially involves three steps: (1) introduction of diversity in the antibody V-genes, creating a 'secondary' library (Fig. 1); (2) selection of the higher-affinity from the low-affinity variants; and (3) screening to allow discrimination of antibody variants with differences in affinity or kinetics of binding. The selection may be chosen to favour kinetic parameters[30] such as off-rate or affinity; this hinges on the use of limited and decreasing amounts of antigen, and on performing the selections in solution rather than by avidity-prone panning. For example, antibodies of the highest affinity can be preferentially selected by using the antigen concentration at or below the desired dissociation constant[30].

The areas that are best targeted for mutagenesis will differ for each individual antibody. Diversity may be introduced more-or-less randomly in the V-genes, as with error-prone PCR, by using mutator strains, by chain shuffling or by DNA shuffling. Chain shuffling of V-genes has been used successfully to obtain a 300-fold increase in the affinity of an anti-hapten antibody, originally isolated from a naive human scFv library[31]. In this experiment, first the light chain and then the heavy chain (without CDR3) were replaced by a repertoire of human partner domains somatically mutated *in vivo*, and from these repertoires the best variants were selected on antigen. A similar approach was applied to antibodies binding to protein antigens, with a more modest sixfold affinity improvement for an anti-c-erbB2 antibody[32] but no improvement for gp120 binding Fabs (Ref. 33).

Alternatively, the antibody's CDR regions may be targeted, using oligonucleotide-directed mutagenesis or spiked oligonucleotides and PCR. With over 100 CDR residues constituting the antigen combining site, a choice will have to be made as to where to start. If the structure of the antibody–antigen complex is known, residues that contact the antigen or may influence other residues contacting the antigen could be targeted. Such residues may also be determined experimentally, by chain shuffling[34], alanine-scanning of the CDR regions[35], parsimonious mutagenesis[36], or modelling. Residues involved in maintaining the main chain conformation of the CDR should be conserved; residues that modulate affinity may be randomized, ideally 4–6 residues at a time to allow efficient sampling of the sequence space. Sequential targeting is preferred, as additive effects of mutants obtained by targeting CDRs in parallel and combining mutations frequently prove unpredictable[37]. Using 'CDR-walking', Yang and colleagues[37] created independent libraries with mutations in one of four CDR regions, and combined the selected variants in one clone. This strategy yielded a 420-fold increase in affinity for the best anti-gp120 Fab.

Can the immune system teach us how to design mutagenesis strategies? *In vivo*, the diversity introduced by somatic hypermutation in the maturation process is mainly located at the periphery of the antigen combining site[38], suggesting that we should target the first and second CDR regions for *in vitro* affinity maturation. However, the best examples of affinity maturation *in vitro,* yielding in some cases picomolar affinity antibodies[34,35,37], highlight the importance of targeting the centre, i.e. the CDR3 of the heavy chain. The powerful *in vitro* maturation procedures described may allow specific introduction and selection of mutations by iteration, in a way not available to, but also not required by, the immune system.

Future prospects

The above mentioned picomolar-affinity human antibodies to viral antigen HIV gp120 and tumour antigen c-erbB-2 are probably the first two examples of antibodies with an affinity and exquisite fine-specificity not previously accessible. We can finally envisage clinical trials with human antibodies that are better than any antibody made before, even better than the ones made by nature.

Although originally intended for the production of fully human antibodies, phage-based methods are creating opportunities for engineering antibody affinity and specificity not previously envisaged. Without doubt, most novel features in antibody engineering will be based on the synthetic antibody concept. Downstream processing and biotechnological production may be facilitated by choosing only those V-gene segments that are expected to be expressed to high levels in the heterologous host. The V-gene building blocks may be modified or selected to incorporate a ligand binding site for purification, a reactive site for introducing probes within the antigen combining site, or CDR-loops with different canonical forms or derived from other species. In addition, the development of 'designer' libraries is envisaged, in which the

building blocks have a propensity for binding to a certain type of antigen by virtue of choice of the CDR-side chains, or provide structurally similar antigen combining sites such as flat surfaces, pockets or canyons.

Affinity maturation strategies need to be developed that allow maturation of selected antibodies in batch mode rather than on an individual basis. Most likely, a mixture of systematic cloning and re-selection methods, or of libraries with carefully pre-mutated building blocks matching the initial design of the diversity of the primary synthetic library, will be used. Eventually, both the selection from the primary library as well as the affinity maturation event may take place in a purpose-built automated machine, possibly the ultimate goal in the biotechnological generation of tailor-made antibodies.

Acknowledgements

I acknowledge my colleagues Dr Simon E. Hufton and Prof. Dr J. W. Arends for critically reviewing the manuscript and for their suggestions.

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Hepatitis C virus diagnostics: technology, clinical applications and impacts

Cathy Conry-Cantilena

Infection with hepatitis C virus (HCV) can cause several significant health problems. Sensitive and specific assays for antibodies to HCV are available to identify and confirm individuals infected with HCV. Evaluation of the clinical impact of HCV infection in a patient includes measuring liver biochemistries and possibly liver biopsy. Molecular assays such as HCV genotype identification and qualitative/quantitative HCV RNA analyses may be valuable in considerations of prognosis and therapy. Further refinement of antibody screening and confirmatory assays and standardization of molecular testing are necessary to optimize testing and thus fully characterize the diagnosis of HCV infection.

In 1989, the primary cause of transfusion-associated, non-A non-B hepatitis was identified using molecular cloning techniques and is now known as the hepatitis C virus (HCV) (Refs 1,2). HCV is a single-stranded positive-sense RNA virus classified within the family Flaviviridae and is global in distribution. Estimates of HCV prevalence in blood donors worldwide range from 0.5 to 8% (Ref. 3).

Although acute HCV infection is most often clinically asymptomatic, health problems may arise years to decades later (Fig. 1). In fact, 80–90% of patients with transfusion-associated or sporadic HCV are unable to resolve the infection[4–6]. The measurement of serum liver enzymes such as alanine aminotransferase (ALT) is used as a simple indicator for hepatitis. An elevated ALT and detectable serum HCV RNA occur early in HCV infection and continue to be found in subsequent tests on the majority of these patients. Antibodies to HCV (anti-HCV) may not be detected, even with the latest commercially available tests, until some time (10–12 weeks) after initial infection. The majority of people who harbor hepatitis C virus infection, identified by blood donor testing, have evidence of chronic hepatitis by liver biopsy. Serious sequelae associated with chronic HCV infection may occur years to decades after the initial infection and include cirrhosis and hepatocellular carcinoma. Presently, therapeutic options for patients with HCV-associated chronic hepatitis and cirrhosis are limited. Interferon-α 2b (IFN) is the only effective medication and only 15–25% of those treated with a standard course achieve a sustained clinical remission[7,8]. In addition, syndromes such as essential mixed cryoglobulinemia, membranoproliferative glomerulonephritis and porphyria cutanea tarda are recognized as extrahepatic manifestations of chronic HCV infection[9].

Several characteristics of HCV impede advances in diagnostic technology. Advanced molecular biologic techniques have permitted the cloning, sequencing and analysis of the HCV genome[1,2]. As yet, the virus cannot be grown efficiently in culture. The chimpanzee is used as a model for infectivity but no simple animal model has been found. In a classification proposed by Simmonds and colleagues, six major HCV genotypes (1–6) along with a series of subtypes (designated by a lower case letter following the arabic numeral) are used to characterize HCV infection[10,11]. Although infection can result from any of these genotypes, the most common genotypes in western nations are 1a and 1b. Considerable sequence variation between HCV genotypes and subtypes, in conjunction with a high spontaneous mutation rate, contribute to the complicated picture of HCV diagnostics and therapeutics. Overall genomic sequence similarity is estimated to be approximately 70–80%. The 5′-untranslated and nucleocapsid regions are relatively well conserved while hypervariable genetic sequences are present in the E1 and E2 domains encoding envelope proteins (Fig. 2). It is this hypervariability that may contribute to the inability of most people to develop a protective humoral antibody response. Infection with multiple HCV genotypes is not common but HCV has been shown to circulate as a heterogeneous population in the form of 'quasispecies' in

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PII: S0167-7799(96)10076-7

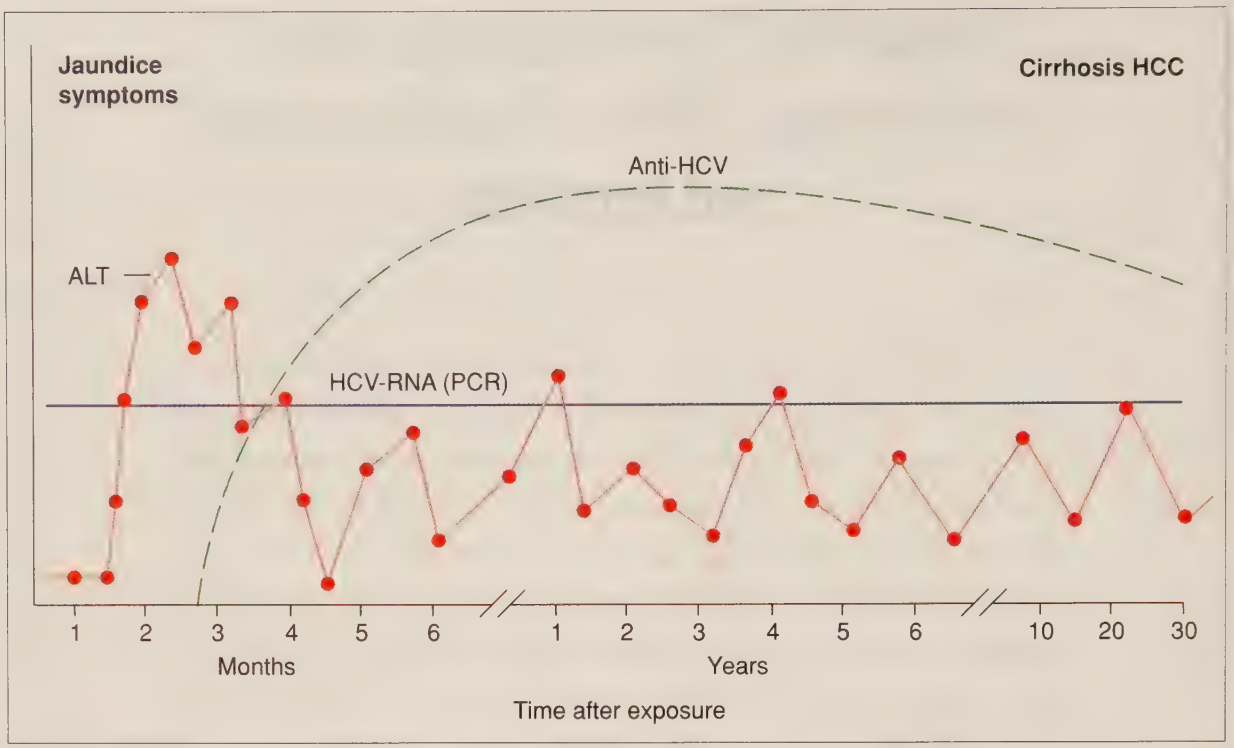

Figure 1

The natural clinical, serologic and virologic courses of HCV infection. HCV RNA is detectable by the polymerase chain reaction (PCR) early in infection and persists in 80–90% of those infected. Serum alanine aminotransferase (ALT) rises 6–8 weeks after infection; at approximately the same time jaundice and symptoms of hepatitis might be experienced. Mild elevations in serum ALT persist in the majority of individuals. Nonprotective IgG antibodies to HCV (anti-HCV), as tested by enzyme immunoassays, develop about ten weeks after infection. Chronic hepatitis with the development of cirrhosis and hepatocellular carcinoma (HCC) may occur after years to decades of infection.

patients years after initial infection[12]. This is a result of sequence divergence, an inherent feature of RNA viral replication. From a virologic standpoint, HCV, like HIV, may exist as a number of variants even within the same patient. Identification of an antibody that neutralizes HCV remains elusive.

However, diagnosis of infection usually depends on the detection of HCV antibodies. Supplementary information can be provided by HCV RNA assays but these are expensive and technically challenging. Depending upon the type of patient undergoing evaluation, antibodies to HCV can be present in the absence of HCV RNA or absent in the presence of HCV RNA. A combination of serum biochemistry, antibody detection assays and molecular techniques to detect viral RNA is most helpful in precisely defining the diagnosis, establishing a prognosis and in monitoring the therapy of HCV infection.

Evaluation of HCV infection: biochemistry and serology

Biochemical evidence of liver disease and indirect measures of infection

Before the availability of enzyme-linked immunosorbent assays (EIAs) for the detection of anti-HCV, indirect or 'surrogate' measures of potential infectivity, using ALT assays and detection of antibody to hepatitis B core antigen (anti-HBc), were employed in the USA to screen blood donors and thus reduce the incidence of post-transfusion hepatitis. The value of ALT assays was based on the pathology of hepatitis while anti-HBc had value for screening out donors with the high-risk behaviors associated with transmitting viruses such as the non-A, non-B hepatitis virus (now known as HCV) and HIV. With the use of these surrogate markers, the rate of hepatitis C virus infection from blood transfusion in the USA steadily declined from 0.45% per unit transfused before surrogate testing, to 0.19% per unit transfused after surrogate testing to 0.03% per unit transfused after implementation of anti-HCV testing[13]. With the evolution of more sensitive and specific anti-HCV EIAs for testing the blood supply, the residual risk of transfusion-acquired HCV infection is probably even lower than this estimate[14]. Although anti-HBc testing of the blood supply continues to be required in the USA because of its small effect in decreasing transfusion-transmitted hepatitis B, the value of routine testing of blood donors for ALT after the introduction of anti-HCV screening was deemed negligible at a National Institutes of Health Consensus Development Conference[15] and its use is no longer mandated.

However, testing for ALT remains important in the clinical evaluation of the patient with hepatitis. It is a widely available, simple biochemical means of assessing liver disease activity and the relative severity of hepatitis. It can be used clinically to segregate those patients whose clinical course warrants intervention

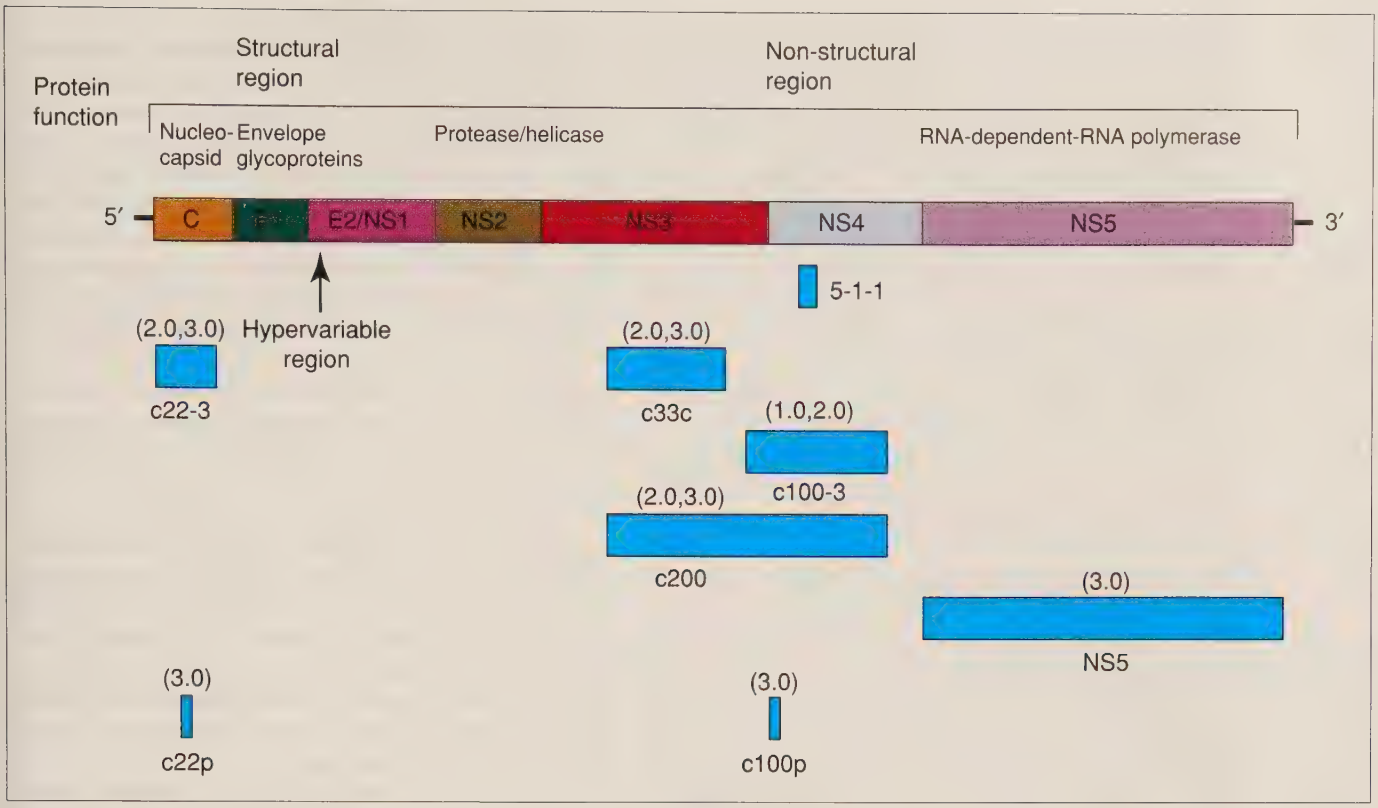

Figure 2

The organization of the HCV genome and its proteins expressed in first, second and third generation HCV antibody assays. HCV proteins are encoded by structural (C = core or nucleocapsid, E1 and E2 = envelope glycoproteins) and non-structural regions (NS1 to NS5) of the genome. Recombinant antigens used in anti-HCV diagnostic assays are designated by 5-1-1, c22-3, c33c, c100-3, c200 and NS5; c22p and c100p have been recently introduced as synthetic peptides. The generations of anti-HCV assays in which the proteins are incorporated are given in parenthesis above the specific protein.

with liver biopsy and possible therapy. For example, an ALT level more than double the normal upper limit may suggest severe inflammatory activity. Normalization of ALT and elimination of serum HCV RNA in response to IFN therapy do not guarantee successful treatment of disease but are often used together to follow patients before, during and after therapy.

A normal ALT level does not eliminate the possibility of liver disease. Even asymptomatic individuals with anti-HCV and normal ALT levels can have chronic hepatitis[16], albeit that the severity of their hepatitis generally does not require therapy. Thus, liver biopsy of these individuals cannot be considered standard practice at this time.

Serologic assays

In 1996, the screening of blood donors for antibodies to HCV infection remains standard practice. Unfortunately, the serology of HCV infection does not follow the classic pattern of IgM response preceding the appearance of detectable IgG. The IgM response may be absent, late or persistent[17,18]. Therefore, diagnosis of HCV infection relies upon detection of anti-HCV IgG and/or HCV RNA. Unlike hepatitis B virus infection, in which viral antigens may be detected in serum during the acute and chronic infectious stages, there is a lack of tests for HCV antigens in sera. HCV antigens may be detected immunohistochemically in specimens of liver tissue after biopsy. HCV RNA screening of the blood supply could provide additional sensitivity and earlier detection of HCV infection but the implementation of molecular technology for routine, large-scale use is not yet technically practical or economically feasible. An array of diagnostic tests exists for anti-HCV screening of blood donors and patients; the latest is described as the 'third generation' of EIAs and confirmatory immunoblot assays.

First- and second-generation antibody screening assays

The structure of HCV genome aids in understanding the evolution of HCV antibody screening and confirmatory assays is shown in Fig. 2. Structural and non-structural proteins are derived from the HCV RNA open reading frame and include those for the nucleocapsid (core), envelope glycoproteins, a protease/helicase and an RNA-dependent RNA polymerase. In the initial cloning of HCV, a single expressed protein (5-1-1) was identified using an immunoscreening technique[1,2]. The c100-3 recombinant protein, the basis of first-generation EIAs (EIA 1.0), was developed using overlapping clones and expressed with superoxide dismutase as a fusion protein in yeast. The ability of EIA 1.0 to detect HCV-infected persons depended upon the population being studied and varied from approximately 60% in blood

donors to 90% in persons with parenteral exposures and chronic hepatitis[19–21]. It was a critical first step in HCV detection. However, EIA 1.0 has limited sensitivity in detecting viral antibody in the acute phase of HCV infection; the average interval from HCV infection to detection of anti-HCV using EIA 1.0 was about 22 weeks in patients with acute hepatitis[22]. Also problematic was its poor specificity, with a large number of false positive reactions given by EIA 1.0 for low-risk populations such as blood donors[23]. Serodiagnosis improved substantially with the development of second-generation assays (EIA 2.0), which employed multiple recombinant antigens[24]. In addition to the c100-3 antigen, EIA 2.0 incorporated the c33c and c100-3 proteins into an expanded nonstructural or composite protein (c200) and also employed an antigen from the core region (c22-3). This combination of antigens resulted in more sensitive detection of anti-HCV (99%) during the acute, serologic 'window' as well as in the chronic phase of infection[20,21].

A reactive EIA screening test should be followed by a confirmatory immunoblot assay. The term 'confirmatory' is not wholly accurate since the recombinant immunoblot (RIBA) employs proteins derived from the same antigenic domains involved in screening EIAs, but uses a different assay technology. RIBA uses HCV-encoded antigens fixed into individual bands on a nitrocellulose strip, which are reacted with patient sera. As with the screening EIAs, these specific tests for confirmation of anti-HCV reactivity also have evolved. First-generation RIBA used two recombinant non-structural proteins (c-100-3, 5-1-1). The more specific, second-generation RIBA (RIBA 2.0) (Refs 25,26) incorporates an additional non-structural protein (c33c) and the nucleocapsid protein (c22-3). A specimen is considered 'positive' (confirmed) if two or more antigen bands are reactive, 'indeterminate' if only one band reacts and 'negative' if no bands react with overlaid serum. HCV RNA is generally detected in 80–90% of blood donor samples[27,28] confirmed by RIBA 2.0. Blood donors with a positive EIA and a negative RIBA result represent persons with a false positive screening test. EIA positive, RIBA indeterminate reactions also occur and are generally directed against the nucleocapsid protein (c22-3) (Refs 24,26 and 29). To determine if these RIBA indeterminate persons are truly infected with HCV, repeat serologic testing and testing for HCV RNA becomes especially important.

From a clinical perspective, the particular anti-HCV pattern seen in the RIBA has no prognostic value with respect to the severity of HCV infection or the potential for clearance of infection. The persistence of anti-HCV antibodies in chronic HCV infection may be diminished in patients treated with IFN but they are not used as a criterion to judge therapeutic efficacy. Patients who have a decreased ability to produce and maintain antibodies, such as those immunocompromized by advanced HIV infection or hypogammaglobulinemia, may have a poorer prognosis, potentially owing to unrestrained HCV replication[30,31].

Third-generation anti-HCV assays

Third-generation anti-HCV assays have been available in Europe since 1993 but only one manufacturer has been granted a licence for the third version of the anti-HCV EIA in the USA. Both types of third-generation assays for anti-HCV (EIA 3.0 and RIBA 3.0) were designed to further improve sensitivity and specificity by adding more antigens to favor specific over nonspecific reactivity. EIA 3.0 uses three recombinant antigens (c22-3, c200 and NS5) derived from four different regions of the HCV genome. RIBA 3.0 employs modified c33c and recombinant NS5 antigens. In addition, two synthetic peptides, c100p and c22p, replace the two recombinant proteins used in RIBA 2.0 to reduce the number of falsely positive reactive sera.

EIA 3.0 is more sensitive and slightly more specific than earlier generation tests[32–34]. In the USA, it is estimated that the risks of HCV transmission using EIA 2.0 and EIA 3.0 are 1 in 92 000 and 1 in 108 000 units of blood, respectively[14]. Both EIA 2.0 and 3.0 are highly specific in blood donors (>99.8%) but the latter has its advantage in blood screening owing to its ability to detect anti-HCV about nine days earlier than the second-generation assays.

Using NS5 in third-generation antibody assays allows for broader detection of HCV-encoded epitopes. However, although HCV-infected individuals occasionally seroconvert first to NS5, detection of anti-NS5 does not appear to be the basis for this assay's increased sensitivity[35,36]. Rather, the differences in sensitivity between the second- and third-generation EIA tests are due to conformational changes in the NS3-derived antigen(c33c) (Refs 33,37).

As with EIA 3.0, the configuration of RIBA 3.0 offers increased sensitivity and specificity compared to RIBA 2.0. Studies have shown that it reduces the number of sera considered anti-HCV 'indeterminate' in HCV RNA-positive persons[38]. However, isolated reactivity of either the modified c33 antigen or NS5-encoded antigen produced a small, but new, population of RIBA 3.0 indeterminate, HCV-RNA-negative individuals. In blood donors' specimens with no detectable HCV RNA, RIBA 3.0 was useful in eliminating many of the c22-3, RIBA 2.0 indeterminate reactions[33]. The use of new antigens such as the E2 protein in future generations of anti-HCV tests might offer higher sensitivity and specificity[39]: an ideal immunoblot might require representation of all the antigenic domains.

In summary, although serologic diagnostic accuracy has greatly improved, anti-HCV assays are still not absolutely dependable in correctly identifying and confirming all cases of infection in all patients. Molecular testing, therefore, plays an integral role in the evaluation of possible HCV infection.

Viral detection by molecular methods

Detection and quantitation of HCV RNA

Although molecular methods for the detection of HCV have not yet reached full maturity, they ultimately

represent the gold standard in HCV testing. Both qualitative and quantitative assessment of HCV RNA in serum may be important in diagnosis, confirmation, prediction of response to and outcome of therapy in HCV infection. Because not all serologically RIBA-confirmed cases of HCV infection are positive for HCV RNA by PCR (polymerase chain reaction), it is unlikely that molecular methods will replace serologic assessment of the patient or donor but rather supplement our diagnostic armamentarium.

Widely used nucleic acid assays include the reverse transcription polymerase chain reaction (RT-PCR) and the branched-chain DNA (bDNA) assay. These assays differ in the way the nucleic acid is amplified for detection. RT-PCR relies on amplification of a viral RNA 'target', while bDNA uses 'signal' amplification to confirm the presence of viral nucleic acid[40–42]. Commercial assays are available for quantitative and qualitative HCV PCR as well as for the bDNA assay. Despite its advantage of great sensitivity, qualitative PCR testing has proved difficult to perform in non-specialized laboratories and is prone to both false positive and false negative reactions[43]. A recent study concluded that the bDNA assay has a narrower linear range for HCV RNA quantitation than quantitative RT-PCR (qRT-PCR)[44]. It was found that although bDNA was both less time-consuming and labor-intense than qRT-PCR, it was also less sensitive. That is, a nonreactive bDNA sample cannot be used as a criterion for the absence of virus especially in patients who might have low levels of viremia.

Useful information may be garnered by the detection and quantitation of HCV RNA using kits for PCR and bDNA. A helpful analogy is the great progress that has been made in the area of quantitation of HIV viremia over the last year. Molecular quantitation of HIV has become a critical clinical tool. The quantity of HIV RNA in plasma has been shown to directly reflect the titer of the virus and to predict disease progression. It will certainly play a role in the optimization of anti-retroviral therapy. Similarly, standardized, accurate qualitative and quantitative assessment of HCV RNA in blood would allow clinicians and researchers to answer similar questions regarding HCV infection. It has already been noted that the larger the viral burden of HCV in serum and liver, the smaller the chance of response to IFN therapy[45]. Further, it could enhance our understanding of HCV transmission patterns and aid in the appropriate counseling of patients. Although the ability of HCV serologic tests, in concert with direct questioning of blood donors, to identify HCV-infected blood donors is excellent, the ultimate gold standard would be the use of a well-standardized method such as PCR for testing the blood supply to close the seronegative 'window' period of infection.

Genotype determination

When HCV RNA is present, genotyping can usually be performed. Various methods have been used to determine genotype. The best method, albeit labor-intense and expensive, is amplification and sequencing of the genome with sequence comparison and phylogenetic tree construction. This method does not lend itself to determination of genotypes in large numbers of patients. A relatively simple method for HCV genotype determination, which has shown good concordance with more complicated methods, is the line-probe assay[11]. It involves hybridizing PCR products from the 5′-untranslated region with genotype-specific oligonucleotide probes immobilized in membrane strips. Other methods include performing PCR with genotype-specific primers or restriction fragment length polymorphism technology. Genotype may be important in the clinical decision-making process because a certain genotype, such as type 1b, may have increased severity of liver disease and a lower rate of response to IFN therapy as compared to others[46].

The future of molecular testing for HCV

What are the problems with making greater use of these clinical tools? At present, commercially available kits for HCV RNA detection and quantitation are relatively expensive. A major difficulty with the use of molecular technique is the lack of standardization between laboratories[43] and interlaboratory studies using commercial kits with a well-defined standard sample of HCV RNA have not yet been performed. Although the design of commercial HCV RNA assays is based on highly conserved regions of the HCV genome, it is possible to underestimate HCV RNA levels of a particular genotype. This has been shown to be true of the bDNA assay but no study of the effect of genotype on qRT-PCR has been reported[47].

Conclusions

Great strides have been made in HCV diagnostic technology in a short time. Laboratory evaluation of a patient or blood donor with anti-HCV should include the combination of liver biochemistries and the recombinant immunoblot assay. HCV genotyping, qualitative and quantitative tests for HCV RNA may be a factor in clinical prognosis and therapeutic strategy. Future directions in diagnostic testing for HCV infection include the refinement of antibody assays and standardization of molecular methods for HCV RNA.

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Applications of flow injection analysis to analytical biotechnology

Peter J. Keay and Yu Wang

Here, we present an overview of the current applications of flow injection analysis (FIA) techniques in the field of biotechnology. Different aspects of this technique that can be applied for specific purposes are discussed with particular emphasis placed on applications in pharmaceutical, food, beverage, clinical and environmental analysis. In addition, the working principles, instrumentation and techniques of flow injection analysis are described.

The determination of chemical parameters is of great use to all kinds of bioprocesses. Online methods for the measurement of important chemical compounds, elements, nutrients, metabolites and products that can be measured in real-time help to further our understanding and control of industrial processes. One solution to measuring such parameters is the use of non-segmented flow injection analysis (FIA). Since the first paper published by Ruzicka and Hansen in 1975 (Ref. 1), FIA has undergone considerable development, with wide applications in biotechnology. FIA techniques have the advantage of being fast, sensitive, stable, accurate and have a small sample volume requirement compared with many other techniques. With suitable arrangement, the technique can be used at- or on-line, allowing real-time measurement of parameters, which until recently have required the back-up of the analytical laboratory; this can then lead to the control of inputs that affect them.

Essentially, flow injection is a means of delivering a sample to a detector, either directly or after mixing with reagent(s). The growing importance of these techniques is reflected in the number of papers published since the

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PII: S0167-7799(97)01005-6

initial publication of Ruzicka and, in particular, in the number of monographs published on the subject[2–6]. This literature covers a wide range of methods, analyses and applications, as shown in reviews of clinical chemistry[7,8], pharmaceutical analysis[9–11], environmental analysis[12–15], food analysis[16,17] and general biotechnology[18–21].

The working principles of the FIA system

The definition of FIA is the mixing of a sample in a tube with specific reagents, within a continuously flowing stream of carrier liquid, while travelling to a detector (Fig. 1). The detector monitors a property of the liquid that undergoes a change according to the extent of the selective reaction that has occurred between the injection of the sample and its arrival at the detector. The principal advantage of this technique is that the incompressibility of the non-segmented flow allows the time interval between injection and detection to be reproduced with high accuracy. This allows: (1) reaction products to be monitored without waiting for a steady-state; (2) a high rate of sampling to be achieved; and (3) reactions that do not go to completion to be employed. The simple form of a conventional FIA system is illustrated in Fig. 2a. The FIA system comprises a carrier stream and reagent propelled by a peristaltic pump injection system – many of these system designs are commercially available. By contrast, a more powerful gas-driven injection system has been developed by Keay *et al.*[22] (Fig. 2b). The system is based on a gas pressure driven FIA technique, with the carrier and reagents being controlled by computer switched solenoid valves. This system has the advantages of being faster, simpler, more accurate and able to be miniaturized. At present it is undergoing commercial development.

Applications of FIA in the food industry

FIA was first applied in the food industry to the analysis of beverages, such as soft drinks, beer, wine and milk, which have normally been limited to the determination of pH, CO_2, sugars, acidity, preservatives and trace metals. The measurement of pH has been carried out by a simple type of FIA in which the sample is presented to a pH electrode. Acidity has been determined by a flow injection gas diffusion technique[17]: the aqueous sample containing sulphite ions is injected into an acid stream where gaseous sulphur dioxide is formed; this diffuses through a gas-permeable membrane into a reagent stream where the product is measured by colorimetry. Carbonate or bicarbonate ions have also been measured in a similar way. Lead and cadmium were measured by combining with dithiazone, followed by solvent extraction and measurement of the colour of the solvent stream[23]. An FIA technique for caffeine determination has also been reported by Osborne and Tyson[17]: a cola sample was injected into a carrier stream which is merged with aqueous ammonia, and the caffeine extracted into chloroform in a solvent extraction module. The absorbance of the chloroform stream is measured. This method can also be used for coffee and tea analysis.

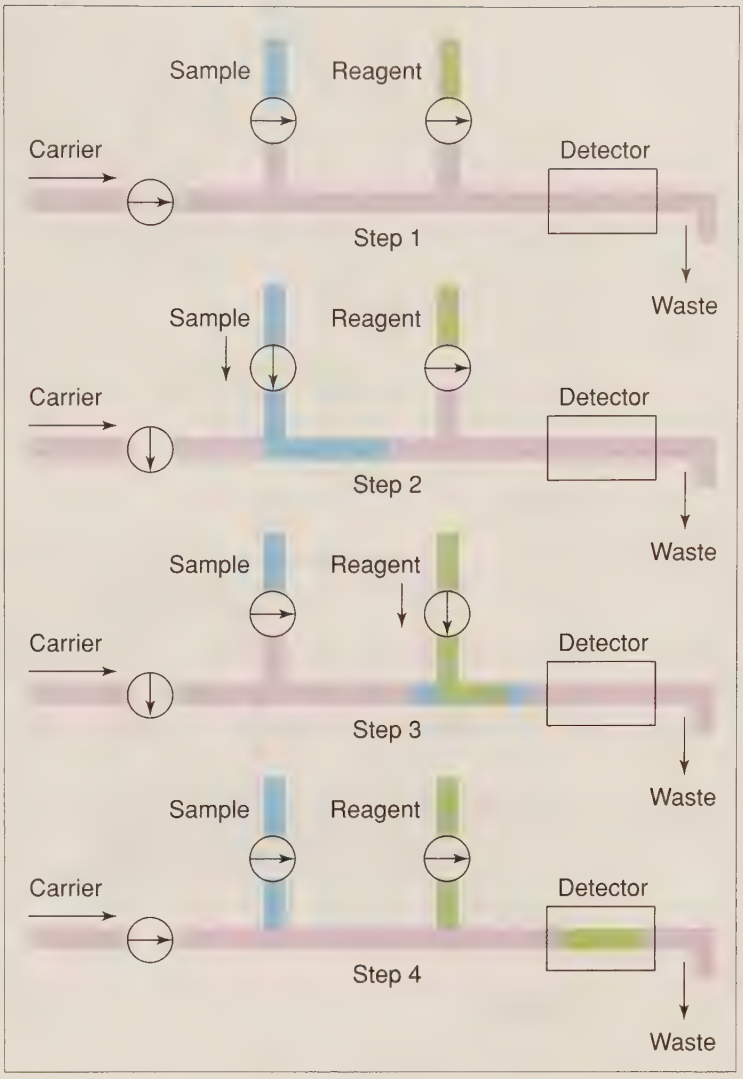

Figure 1
The principle of a single-channel flow injection analysis operation. Step 1: the carrier is injected with the carrier valve on, and sample and reagent valves off. This is to clear the tube and obtain a detector base line reading. Step 2: the sample valve is switched on to inject the sample into the tube with the carrier and reagent valves off. Step 3: the reagent is injected into the arriving sample pellet with the carrier and sample valves off. Step 4: after a predefined reaction time (stopped flow if necessary), the carrier valve is switched on again to push the coloured material towards the detector (spectrophotometer) for measurement.

In addition to the above applications, FIA techniques have been applied to more specific analyses of beer, wine and milk. Automatic FIA systems based upon a zone sampling technique have been described for the determination of calcium and magnesium in wines by atomic absorption spectrometry (AAS), and sodium and potassium by flame photometry[24]. The measurements of volatile acids, chlorides and high molecular weight compounds in beer have been reported by many researchers[25–27]. Bitterness in beer is determined by solvent extraction from an acidified carrier stream and measurement of the absorbance of the solvent stream in UV. A method for simultaneous quantitation of citrate and isocitrate in citrus juice using an FIA system has also been developed[28]: the relative standard deviations for ten successive injections

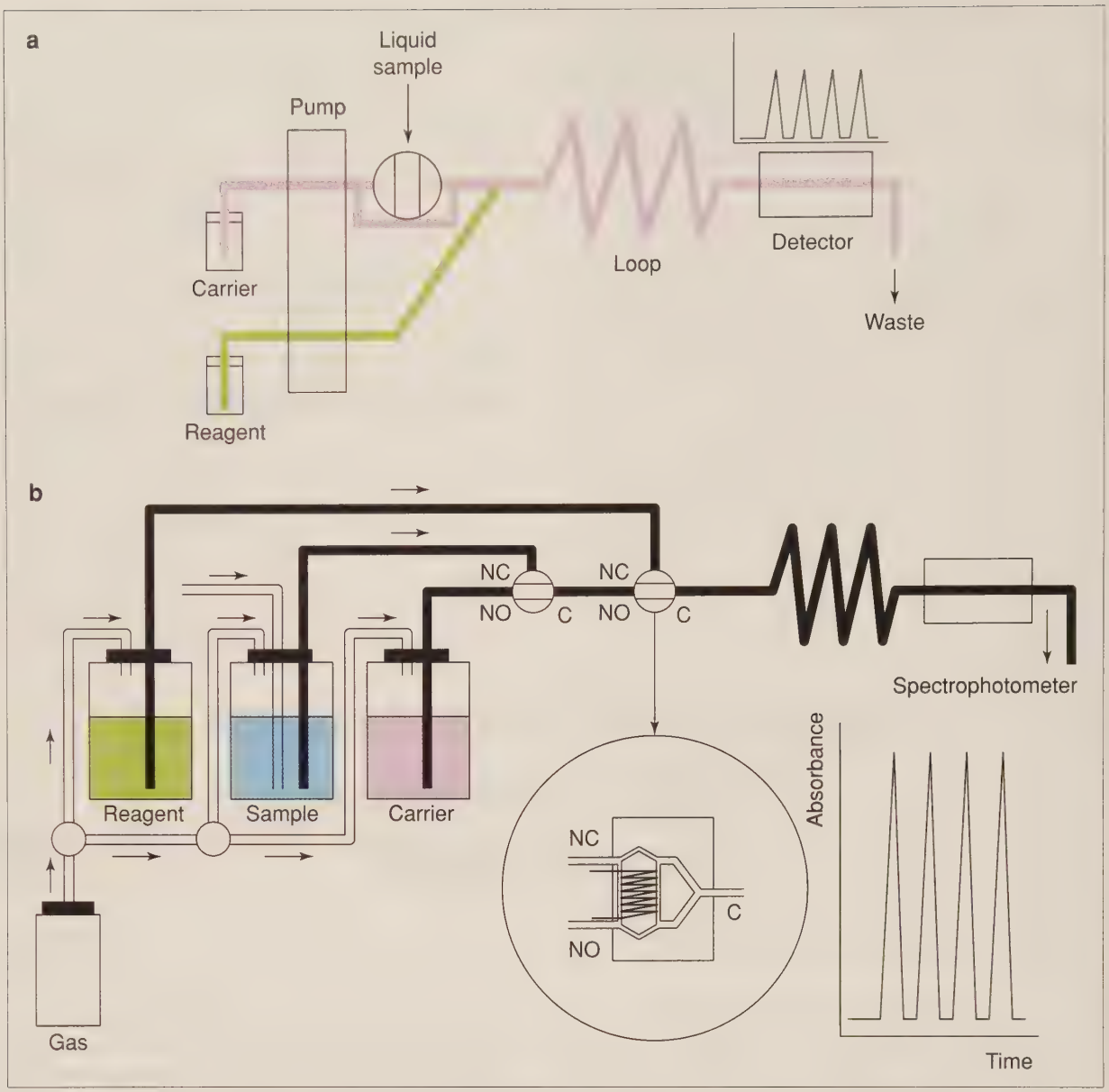

Figure 2

(**a**) General arrangement of a conventional FIA system, showing its essential components: peristaltic pump, injection valves, loop and detector. (**b**) Flow diagram of a gas driven FIA system and solenoid valve connections. Abbreviations: C, common; NC, normally closed; NO, normally open.

were < 2%. Cliffe[29] also reported the measurement of phenolic content in natural juices by an FIA system.

The applications of FIA techniques in solid food analysis have been described by many researchers including: (1) the analysis of potassium bromate in flour[30]; (2) total volatile acids, which is a well-known indicator for the decomposition of seafood products[31]; (3) flour extraction of vitamin B1 (Ref. 32); and (4) an assay of cereal α-amylase[33]. However, all these applications have involved the preparation of solutions from solid food samples and are normally carried out by acid digestion, aqueous extraction or dry ashing, followed by dissolution of the ash.

Interestingly, FIA has recently been used to detect *Escherichia coli* in artificially contaminated food samples[34]. The system successfully detected *E. coli* in < 30 min and was reusable for at least 300 repeated assays. A low-cost chemiluminescence detector with a photodiode for flow injection analysis of L-lactate in food has been described[35] and shown to be a simple, easy to use instrument for quality control.

FIA has also been used for the determination of formaldehyde in frozen fish, offering another example of its versatility[36]. This system was furnished with a gel-filtration chromatography column for online removal of the proteins from the extract before the enzymatic analysis was performed; it is much faster and less affected by interference compared with other formaldehyde determination methods.

FIA for pharmaceutical determinations

Bioprocesses are generally insufficiently controlled, as online measurements of relevant biological variables are difficult to obtain[18]. Reliable quantification of

physical and (a few) chemical variables is usually possible, but the determination of biological conditions is very demanding. Therefore, measurement of the important physiological parameters has usually been achieved by offline methods. Some enzymatic sensors are available for on-line measurements, but the disadvantages of short life, instability and the fact that they cannot be autoclaved limits their application. The use of FIA for on-line measurements in the pharmaceutical industry promises to be important in fermentation process development and optimization. By using FIA techniques, a process monitoring system can be constructed which, in addition to high sampling frequency and high reproducibility, offers a small sample volume requirement, low reagent consumption and real-time continuous monitoring. An FIA system can easily be integrated into biotechnological processes and is well suited for fermentation or downstream process monitoring with close-loop control. For online fermentation monitoring and control, FIA is undoubtedly versatile and is easy to set up compared with other techniques, such as mass spectrometry and liquid and gas chromatography. It also allows the combination of a variety of analytical and sample pretreatment devices. Detection systems used with FIA are UV/visible/NIR spectrophotometry, chemiluminescence, fluorometry, mass spectrometry, atomic absorption, flame emission photometry and various electrochemical techniques[37].

During the past few years, several applications of FIA systems have been developed for online fermentation monitoring and control[38–44], and online protein detection in biotechnological processes[45–47]. Nielsen[40] has developed a method for semi-online monitoring of several variables in lactic acid fermentation, which has been used to monitor transient responses in continuous fermentation and underlines the effectiveness of FIA for online monitoring. Measurement of glucose and lactate by applying FIA to the study of bacterial and yeast fermentation, especially the product formation pattern at very low glucose and lactate concentrations, has been described by Benthin[48]. The limits of determination in aqueous solutions were 50 μg l^{-1} of L-lactic acid and 150 μg l^{-1} of L-glucose.

The continuous and reliable determination of substrates in media originating from fermentation broth is possible when continuous sample pretreatments are integrated into the corresponding FIA system[49]. An FIA system with immobilized dehydrogenase, which included sample pretreatment by means of a continuously working filtration unit for the elimination of microorganisms, was demonstrated in the parallel determination of glucose and ethanol during a baker's yeast fermentation[50]. Other applications include on-line monitoring of glucose and lactate concentrations in animal cell culture[42,51] and the continuous determination of pullulan in the presence of glucose in a filtered fermentation broth[52]. The parallel analysis of glucose and pullulan in one sample took ~10 min – compared with the 2 h needed for analysis with conventional methods, the use of FIA was a great improvement.

Other enzymatic analyses using FIA that have been described are phenylalanine[53], L-lysine[54], monoclonal antibodies[55], β-galactosidase[56] and cellobiose dehydrogenase activity in fermentation samples[57]. A method for the kinetic determination of four clinically significant enzymes with fluorescence detection has also been reported[58]. Measurement of the activities of formate dehydrogenase and L-leucine dehydrogenase by FIA in downstream operations of *Candida boidinii* and *Bacillus cereus* fermentations[46] showed that the reproducibility and statistical precision of data was much better than that resulting from the manual assays. However, a method developed for online analysis of β-galactosidase[56] in downstream processing suffered from short-term stability due to the reduction in enzyme activity with time. Consequently, this system is not suitable for medium- or long-term online analysis.

Generally, all FIA systems currently used for online fermentation monitoring and control are driven by peristaltic pumps, which result in slow injection. In addition, the accuracy and flexibility of the system is not completely satisfactory. Thus, FIA technology still needs to be improved to meet the requirements of online fermentation monitoring and control. A new gas-driven FIA system for these applications has recently been described by Keay[59,60]. This computer-controlled multichannel FIA system was set up to monitor flux of glucose, ammonia, lactate and lactate dehydrogenase in a mammalian cell culture by means of enzymatic assays. The system response was fast and yielded a large number of reliable and precise analytical results with a variability in ten replicates of < 0.1%.

Applications of FIA to environmental analysis

Since its advent, FIA has been recognized as a very powerful automatic analysis technique in environmental analytical chemistry. Its capacity for handling large numbers of samples, high sampling frequencies and flexibility for adaptation to different types of analyses are responsible for its widespread use in environmental monitoring. The gas-driven FIA system[59,60] is suitable for miniaturization and portability, and work is being undertaken to achieve these objectives. These features will make the system valuable to environmental scientists and authorities.

A number of methods have been developed for the control of different pollutants. However, it should be emphasized that most of the applications involve the determination of pollutants in water samples, for example, the determination of copper, lead, cadmium and manganese in natural water sources using online ion exchange preconcentration techniques[61]. The relative standard deviation was ~2.0% (n = 11) at a sampling frequency of 45 per hour with a 20-fold enrichment of the original sample. Similarly, a method for the determination of lead ions in water samples has a detection limit of 1 μg l^{-1} with a relative standard deviation of 5.2% at the 20 μg l^{-1} level[62]. Another recent application of FIA in environmental analysis is

the determination of 8-hydroxyquinoline (oxine) in water samples at different pH values[63].

A single-channel FIA system for the determination of chloride in soils was developed using a tubular silver ion selective electrode with a homogeneous crystalline membrane of Ag_2S as a potentiometric detector[64]. This system allows the measurement of chloride in soil extracts from 1 to 100 ppm. The sampling frequency was from 6 to 160 samples per hour with standard deviations < 4.8%.

At present, new configurations of the FIA system are being designed for different applications in the environmental field. However, the trend is heading towards the development of an automated portable FIA system for online analyses, especially for the water industry.

FIA in clinical chemistry

Clinical applications of FIA have not gained wide acceptance in hospitals and clinical chemistry laboratories. This is mainly due to the fact that clinical analysis generally requires several different determinations to be carried out on each sample – current commercially available FIA systems do not meet this requirement. However, this problem of simultaneous analysis has been solved[22,59,60] and FIA might begin to play a major role in clinical chemistry. This is particularly true where analysis is required at the point of patient care.

In spite of this, many FIA methods have been developed for clinical applications. A highly selective, rapid and direct amperometric method based on the formation of a complex between tin(II) and 8-hydroxyquinoline, has been reported for the determination of trace levels of tin(II) in dental gels[65].

FIA has also been used to monitor uric acid and glucose levels in human serum[66]. The oxidase and peroxidase enzymes were physically co-immobilized in a sandwich type reactor and a serum sample was injected; the reduction in current, resulting from the enzymatic reactions, was then measured. The results obtained were in fairly good agreement with those obtained using conventional hospital laboratory methods.

Other methods include a system combined with dual biosensors for the determination of glucose in clinical applications[67] and the determination of lactate in serum using a lactate amperometric biosensor[68]. Results obtained by the FIA for lactate compare well with those obtained by a standard enzymatic colorimetric assay and a high sampling frequency was obtained (70 samples per hour). However, after one week of continuous use in the FIA system a 75% decrease in biosensor sensitivity was observed.

An FIA system has recently been developed to examine the relationship between DNA damage, drug resistance and cell survival[69]. Drug concentrations as low as 5×10^{-11} M could be measured by FIA. The method also allows assessment of cross-resistance/sensitivity. The speed, sensitivity and reproducibility make FIA a good technique for routine monitoring of the tumour cell response to DNA damaging agents.

Conclusion

In the past few years FIA has made numerous contributions to biotechnology, with many new configurations of FIA systems and online separation techniques having been evolved. New detectors and control software have been used in conjunction for a wide variety of analyses. The variety of systems touched on in this review demonstrates the growing use of flow injection as a delivery system for samples obtained from bioprocesses during cultivation, upstream and downstream processing, in clinical analysis and in environmental chemistry and the food industry. The flexibility of FIA in combination with a variety of detectors or advanced techniques is clearly established. However, there are still several undesirable aspects in conventional FIA which limit the application of the technique. These include: poor flow characteristics, mostly due to the peristaltic pumps which result in long dispersion tails and hence reduce the system reproducibility; the difficulty of changing injection volumes; the difficulty of interfacing to other systems; and finally, the complexity and expensiveness of most commercial systems.

Current efforts in this area are aimed at the development of completely automated, portable and versatile systems. These systems will have user friendly software for rapid, real-time, continuous measurements of many parameters. In the near future, miniaturization of FIA systems and their combination with portable robust detectors will have a great impact on biotechnology.

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Analytical biotechnology

Articles on the following topics have been commissioned for *Trends in Biotechnology*.

•Atomic force microscopy in analytical biotechnology
•Expanded bed adsorption in industrial bioprocessing
•MEMS for biotechnology
•Ultrasonic separations in analytical biotechnology
•Flow cytometry in bioprocess control
•Biomass measurement on-line
•Dielectrophoresis in analytical instrumentation development
•Ultrasensitive immunoassays: medical and environmental applications
•Biotechnological application of SPR
•Assays for HTS

More methods in molecular biology

DNA Cloning: Expression Systems, a Practical Approach (2nd edn)

edited by D. H. Glover and B. D. Hames, Oxford University Press, 1996. UK£25.00 (149 pages) ISBN 019 963 4785

The problem with trying to review a book dealing with laboratory methods is that it is similar to deciding what a culinary dish will taste like just from looking at the recipe! Clearly, unless one tests each and every protocol, it is very difficult to predict how useful such a volume will really be to the interested scientist. Fortunately, the *Practical Approach* series has a distinguished track record in presenting laboratory methods in a format that is both readable and of value at the bench. In this second edition of *DNA Cloning*, four volumes have been assembled: the first deals with Core Techniques; the second is the subject of this review and concerns Expression Systems, in the third and fourth volumes Complex Genomes and Mammalian Systems, respectively, are considered.

There is a tendency for books to increase in size over the years; new methods are introduced in a field and authors are reluctant to jettison the tried and tested protocols which may no longer be so popular. In *DNA Cloning*, the editors have resisted this temptation and left each volume a reasonable length. However, my personal preference would be for a ring binder format rather than traditional soft-back. If a book of method is truly to be a laboratory manual, then it should sit conveniently on the bench and not curl up in the middle!

Expression Systems presents a concise collection of methods that will be of great use to the modern molecular biologist. This is an area which can be a maze for the uninitiated, especially with such a bewildering choice of expression vectors currently available. At first the subject matter of this volume appeared to be a little confusing. The first three chapters presented no problem and are entirely appropriate in this book; these include descriptions of expression vectors, plasmid expression vectors and protein purification from *Escherichia coli*. The latter section is particularly useful since this is usually the most difficult part of any expression project. It also gives an excellent account of the use of affinity handles or epitope tags for the isolation of recombinant proteins. However, chapter 4, the production of monoclonal antibodies using proteins expressed in *E. coli*, made me wonder whether the editors had mixed up their manuscripts. What was a description of monoclonal antibody production doing in a volume on expression systems? After some consideration, however, this makes more sense. The production of antibodies using recombinant protein as an antigen is a logical progression from the methods described in the previous chapters. My one criticism is that I do not think that it was really necessary to describe the production of recombinant protein in bacteria, since this had already been adequately covered.

Chapter 5 sees the transition to the first of the eukaryotic expression systems described in this volume. This description of yeast expression systems is succinct but comprehensive. There are some books which are entirely devoted to this topic, so the authors have done well to condense a mass of information into such a short section. The yeast theme is continued in the next chapter, where the two-hybrid system is described. This powerful technique for studying protein–protein interaction might not be expected in such a volume. Although most expression systems are generally used to isolate large quantities of pure protein and the yeast two-hybrid system is clearly not intended for this purpose, its use is becoming so widespread that it is appropriate to include it here.

The final chapter in this volume contains a description of the baculovirus expression vector system. I suspect that the editors were not quite sure where to include this topic in the *DNA Cloning* series. It could just have easily been included in the final volume on Mammalian Systems, but would have violated the taxonomy. This chapter includes most of the salient points about the baculovirus expression system, but does omit a description of the recently introduced method for isolating recombinants using bacteria to maintain the virus genome. This method is rapidly assuming prominence and it is a pity that it was not summarized.

The presentation of the *Practical Approach* series has been improved over the years. Each of the submitted chapters follows a common format, while still allowing scope for individual author style. Sufficient theory is given to understand each of the systems and discrete protocols are highlighted by a box format containing all of the reagents required for the experiments. Each step in a protocol is defined by a number, so the reader is not lost in a sea of text. A further innovation which might have been useful would be to provide a common plasmid drawing programme to each of the authors, to standardize the format of each chapter.

Expression Systems will be a very useful addition to the bookshelf of any laboratory concerned with molecular genetics. It is comprehensive without being too long and highlights the important systems for producing recombinant proteins. The perfect expression system has yet to be developed, so most workers have to pick and choose their methods carefully. This book provides sufficient detail for bacterial, yeast and insect systems to make it possible to try all three for synthesizing their favourite protein.

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Engineering improved mycoinsecticides

Intense interest in the scientific community and among the public is being focused on the biological control of insects due to increasing problems with reliance on chemical pesticides. Widely used in some less developed nations, entomopathogenic fungi have recently also assumed importance in US agriculture and household entomology. They are of special relevance for biological control as: (1) the approximately 1000 known species are key regulatory factors in insect pest populations; and (2) they infect insects by direct penetration of the cuticle and thus provide the only practical means of microbial control of insects that feed by sucking plant or animal juices, and for the many coleopteran and acridid pests, which have very few known viral or bacterial diseases[1–5]. In addition, the imperfect fungus *Metarhizium anisopliae* was registered by the USA Environmental Protection Agency in 1993 for cockroach control and in 1995 for termite control, and registration packages for use of *Beauveria bassiana*, another imperfect fungus, were recently approved for grasshoppers and whiteflies. *Verticillium lecanii* is registered in Europe, and there is interest in it in the USA, particularly for aphid and whitefly control[5].

Biological control experiments with traditional biopesticides (viruses, bacteria and fungi) have often produced inconsistent results, and the slow speed of kill compared with chemical insecticides has deterred commercial development. Consequently, any consideration of the suitability of a fungus for commercial purposes inevitably leads to the possibility of improving its performance, i.e. by incorporating more-toxic modes of action and increasing kill rates. Improvements have been attempted through parasexual crossing and protoplast fusion[6] but genetic engineering, by directed addition of one or more genes coding for pathogenicity determinants, provides the most targeted and flexible approach to alter the physiology of entomopathogenic fungi. This will produce combinations of traits that are not readily identifiable in nature without the co-transfer of possibly undesirable linked characteristics. Furthermore, entomopathogenic fungi represent an unconsidered, and therefore untapped, reservoir of pesticidal genes for the production of advanced engineered pesticides and plant products; an important consideration, given that the lack of 'useful' pesticidal genes for transfer has been a major constraint in the implementation of biotechnology in crop protection[7].

Cloning of genes from entomopathogenic fungi

Until recently, recombinant DNA techniques had no application in a strain improvement program involving entomopathogenic fungi because there was no information available as to the nature of the genes that control either pathogenesis or specificity. Our overall cloning strategy has been to isolate genes that are specifically expressed during invasion processes, as amongst these we hoped to find genes that have a key role in allowing the pathogen to establish itself in the insect host. We hypothesized that these genes would be regulated in response to either physical or environmental variables associated with the host environment, and so we isolated genes upregulated in response to a hard hydrophobic surface inductive of the infection structures required for cuticle penetration[8] or during growth on cuticle, as this is initiated by transcriptional activation of a large number of genes[9,10]. To provide a rigorous double blind approach, we used differential hybridization and display techniques to isolate infection-regulated genes without making any assumption about their products.

The first pathogenicity related genes cloned from *M. anisopliae* were obtained by probing a cDNA library to identify members whose mRNA showed increased accumulation during nutrient stress and/or in the presence of cuticle that induces the fungus to be a pathogen[11,12]. This provided the *Prl* protease gene, a previously suspected pathogenicity determinant, and four other clone families not previously suspected of being involved in pathogenicity[11].

We have added to our library of putative pathogenicity-determinants and stage-specific proteins with reverse-transcription differential-display PCR. We used this method to compare mRNA patterns from non-differentiating germlings (-appressoria), differentiating germlings (producing

Table 1. *Metarhizium anisopliae* genes cloned using RT-DD-PCR and their homologous genes from other organisms

Putative toxins	Homology
Trypsin/chymotrypsin inhibitor	Various plants
Toxin	Sea anemone neurotoxins
Toxin	*Acanthamoeba* hemolysin toxin
Hydrolytic enzymes	
Carboxypeptidase	*Homo sapiens* and *Rattus norvegicus*
Subtilisin-like proteinase	*Metarhizium* and other pathogens
Signal transduction	
Protein tyrosine phosphatase	*H. sapiens and Mus musculus* (growth control)
Serine/threonine protein kinase	*Saccharomyces cerevisiae* (STE 20) (morphogenesis)
Protein kinase	*S. cerevisiae* (DAL 81) (transcription activator protein)
Protein kinase	*S. cerevisiae* (CDC 7) (cell cycle control)
GTP binding protein	*S. cerevisiae*
Thymidine kinase	Herpes simplex virus

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PII: S0167-7799(96)10071-8

infection structures), mycelia grown in nutrient-rich media (non-inductive) and mycelia grown on insect cuticle (inductive). Following screening of over 14 000 mRNA species, we isolated 44 developmentally regulated cDNAs; 15 of these have been partially or completely sequenced and eight show sequence similarities to known entities (Table 1). Some of these genes encode enzymes and toxins with demonstrated targets in insect hosts. These results are consistent with biochemical studies showing that *M. anisopliae* produces a mixture of compounds that affect a number of biochemical/organ systems within the target insect[4,8,10].

Construction of an improved mycoinsectide overexpressing a toxic protease

One of the cloned genes encodes a subtilisin-like protease (designated Pr1) which solubilizes the proteinaceous insect cuticle, therefore assisting penetration of fungal hyphae and providing nutrients for further growth[11,13,14]. Pr1 production by *M. anisopliae* is under the dual control of a general carbon catabolite repression/derepression mechanism and a carbon source (cuticle) specific induction mechanism[15]. As penetration involves the induced expression of Pr1, we hypothesized that constitutive overexpression of this gene could provide a direct strategy for engineering enhanced virulence.

Additional copies of the gene encoding Pr1 from *M. anisopliae* were inserted into the genome of *M. anisopliae* under control of a constitutive *Aspergillus nidulans* promoter such that Pr1 was constitutively overproduced in the hemolymph of the tobacco hornworm *Manduca sexta*, activating the prophenoloxidase system. The combined toxic effects of Pr1 and the reaction products of phenoloxidase caused larvae challenged with the engineered fungus to exhibit a 25% reduction in time of death and reduced food consumption by 40% compared with infections by the wild-type fungus[16]. In addition, infected insects were rapidly melanized and the resulting cadavers were poor substrates for fungal sporulation (Fig. 1). Thus, environmental persistence of the genetically engineered fungus is reduced, providing substantial biological containment. These features are also consistent with the recent emphasis on using mycopathogens as contact insecticides, maximizing the kill from the initial application of the fungus in the same way as with a chemical insecticide[1].

Figure 1

The effects of protease overexpression on insects. *Manduca sexta* killed by the wild-type strain (left) and *gpd*-Pr1-4, a recombinant strain expressing Pr1 under the *Aspergillus nidulans gpd* promoter. Following death, hyphae of the wild-type strain re-emerge through the cuticle and produce the characteristic green spores of *M. anisopliae*. By contrast, insects killed by *gpd*-Pr1-4 show considerable blackening, and were very poor substrates for sporulation. Insects were inoculated by dipping in conidial suspensions (1×10^6) and incubated for 10 days in a moist chamber.

The protease itself provides a novel mode of action (activation of prophenoloxidase) for combating potential resistance development in selected pests when expressed in an appropriate fungal or insect virus. Novel features of Pr1, which distinguish it from proteases produced by saprophytes, include its resistance to proteinase inhibitors (serpins) in hemolymph (R. J. St Leger, unpublished data) and its retention of activity in a melanizing milieu[17]. These pathogenic specializations are symptomatic of the fact that entomopathogenic fungi may have spent millions of years of evolution refining chemicals that subdue their hosts. This suggests that the multifarious secreted products produced by entomopathogenic fungi themselves are choice candidates for their genetic improvement. Besides secreted products, directed changes to alter virulence could result from manipulation of nearly every aspect of fungal development biology. For example, some genes encode receptors that detect either directly or indirectly, the presence of the host, e.g. genes for a GTP-regulated adenylate cyclase, tyrosine protein kinases, serine and threonine protein kinases and phosphoprotein phosphatase[8]. These act to change second messenger levels or are themselves activated by second messengers to trigger differentiation of infection structures and production of generic pathogenicity genes[8].

Future prospects

This is a very exciting time for studies on entomopathogenic fungi. We are entering an era when techniques for isolation, identification and subsequent manipulation of expression of individual genes implicated in the disease process will allow the production of transgenic fungi with improved pathogenic qualities and.

hopefully, generate a wider interest in fungi as sources of pesticidal genes. To date, fungal genes have played little part in the implementation of biotechnology in crop protection, which is surprising given the dazzling array of metabolites fungi produce.

A very profitable direction for research could be to use genes encoding the battery of hydrolytic enzymes, toxins and other anti-insect components produced by various entomopathogenic fungi to improve the performance of non-fungal pathogens. The potential of using baculoviruses for insect pest control has long been recognized as they have minimal environmental impact and high target-specificity. An important goal of genetically engineering these viruses has been to improve their ability to kill target insects rapidly. The primary limitation in this area has been the availability of pesticidal genes[18]. Fungi are much more amenable to molecular analysis than alternative sources of toxins, e.g. arthropods, and the screening of fungal genes encoding fast-acting proteins could greatly increase the scope of such studies. We have initiated such studies by introducing the *Pr1* gene into the *Autographa californica* nuclear polyhedrosis virus to produce Pr1-Baculovirus Expression Vector (BEV). Time to death was reduced by about 50% compared with controls when Pr1-BEV was injected into the hemocoel, indicating that the recombinant Pr1 retained its toxic capacity[19].

An alternative strategy is to incorporate fungal genes directly into the plant genome so that the insect will be forced to encounter the gene product when feeding or interacting with the specific host plant. Future success in developing transgenic plants will benefit from the availability of as wide as possible a range of suitable genes. We have recently entered into a collaboration with R. Dixon (Noble Foundation) to test the utility of using selected *Metarhizium* genes in plant systems.

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Meeting report

Biotechnology Down Under

Early international symposia in biotechnology concentrated largely on the control of microbial reactions in fermenters – the production of antibiotics, the accumulation of metabolic intermediates, sensors to measure critical nutrients, or techniques to provide optimal physical conditions for growth and product recovery. The expansion of the scope of biotechnology was clear from the material presented at a recent meeting*. In addition to the sessions on traditional topics, the symposium had sessions devoted to gene therapy, genetically engineered vaccines and food and agricultural biotechnology, the latter areas perhaps reflecting the host country's interest in primary production and food processing. Some 1250 people attended, with about half coming from 49 countries other than Australia.

*The meeting '10th International Biotechnology Symposium' was organized by the Australian Biotechnology Association, and was held at Darling Harbour, Sydney, NSW, Australia, 25–30 August 1996.

Structure of the biotechnology industry

To supplement the specialist topics dealt with in twelve symposia (presented in four concurrent sessions) the organizers invited experts to provide an overview of different aspects of the biotechnology industry in three plenary sessions. The opening plenary lecture was given by William Rutter (Chiron Corporation, Emeryville, CA, USA) who presented his views of the industry and its future directions. He indicated that in the USA alone there are 250 major companies in the biotechnology field, with a total capital of US$33 billion, which is dominated by six companies with a collective capital of US$1 bn. Worldwide, he estimated the capital investment in biotechnology to be about US$55 bn, and suggested that the health care industry absorbed about 7% of this. He observed, however, that there was relatively little effort directed towards the prevention of disease; rather, effort was directed to pharmaceuticals and diagnostics.

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PII: S0167-7799(96)30029-2

He observed that the industry is very mobile. Large numbers of small start-up companies have a life span of 2–3 years; then they either merge with larger companies or die. He also observed that the large companies in the health care industry were increasingly identifying companies or sectors with special skills, and buying in those skills or component compounds.

He also saw a move away from the control of disease with a single agent to more precise management by a combination of therapies. This requires the response of the host to the agents to be carefully monitored during treatment to ensure optimal results. The need for continuing research into this facet of therapy was illustrated by an example from the difficult area of the control of viral infections. From HIV/AIDS research it is clear that cases with low viral numbers progress to AIDS slowly, by contrast with those with high numbers where progression is rapid. It is also well established that a class of white cells in the blood (CD4 T-cells) are depleted in AIDS cases, making the patients prone to other infections as these cells are essential to normal antibody formation. In cases where therapeutics control viral numbers to low levels, the number of CD4 cells does not rise. However, combined therapy with interleukin 2, which raises CD4 numbers, and drug therapy, which reduces viraemia, greatly prolongs survival.

Rutter saw a major new field arising from the development of oligochemistry to synthesize a wide range of organic compounds based on the mimotope technique originally developed to synthesize families of peptides. These large arrays of oligo compounds must be screened for activity and robotics are an essential component of this new route to pharmaceutically useful products. Another significant developing area is the use of 'naked' DNA. This may serve as an alternative to whole cells as a vaccine, or as a means of introducing DNA coding for cytokines in cancer therapy, or for a gene that is deficient in the patient (cystic fibrosis). This requires the DNA to be protected from nucleases, and the design of ligands to which the DNA can be bound and protected is now being actively pursued.

Tapping biodiversity products

Teruhiko Beppu (Nihon University, Fujisawa, Japan) discussed ways of seeking new metabolites from organisms from extreme environments, as these organisms are either non-culturable or are difficult to grow and have not been studied extensively. It is likely, however, that their enzymes and metabolites will have the valuable attribute of tolerance of extremes of temperature or pH. With PCR technology, the genes of non-culturable organisms can be amplified, cloned and studied for useful products or reactions. Beppu went on to discuss ways in which organisms not currently known to produce useful secondary metabolites might be induced to do so. This arose from the observation that secondary metabolites and changes in morphology of *Streptomyces* sp. occurred simultaneously. In turn, this led to the discovery of a protein (A-factor) that was part of the complex regulatory control of secondary metabolites, as well as differentiation. When the A-factor gene was mutated so that its ability to bind to a specific DNA-binding protein was defective, the yield of antibiotic was increased 10-fold. Beppu believes it will be fruitful to insert the A-factor gene or similar regulatory systems into other species to uncover novel secondary metabolites. He also described a thermophilic symbiotic pair of organisms (*Symbiobacterium thermophilum* and *Bacillus* strain S), one of which possessed a thermotolerant tryptophanase, which was cloned and expressed in *E. coli*. Such symbionts are difficult to work with and so have been largely ignored. They are thus worthy of exploration for novel products.

Agricultural biotechnology

Many plants can now be regenerated from tissue culture. Genetic engineering allows foreign genes of agronomic interest to be introduced into tissue cultures and the transformed cells regenerated to yield crop plants with novel genotypes. For example, a gene from *Bacillus thuringieneis* codes for a protein (Bt) toxic to caterpillars; this gene has been introduced into cotton and a variety of horticultural crops. Jim Peacock (Division of Plant Industry, CSIRO, Canberra, Australia) outlined strategies to manage the growth of Bt-cotton to minimize the likelihood of insects developing resistance to the Bt toxin. The first cultivars of Bt-cotton were registered in Australia in 1996, with restrictions on the regions where Bt-cotton could be grown and with a requirement to provide refuge areas of conventional cotton. These areas will yield Bt-sensitive, homozygous moths to mate with any resistant moths emerging from the Bt-cotton. (Moths must be homozygous for resistance to have the Bt-resistant phenotype.) The registration will be reviewed in five years' time. He indicated that pyramiding of other Bt-genes into cotton was in progress to assist in delaying development of Bt resistance, and that agronomic measures for total pest management were being implemented. The reported inability of Bt-cotton to control insects was contested by Fred Perlak (Monsanto Cerogen, St Louis, MI, USA). He explained that last season had been particularly favourable for insect attack, and that whereas Bt-cotton had been sprayed once or twice, conventional plants required five or six sprays to control infestation. Another approach to the control of caterpillars may be possible, should Bt resistance develop. Three genes from an insect-specific virus have been identified. These have been successful in protecting tobacco from larval attack, and it is hoped to transfer these genes to cotton.

Opportunities for improving the biological value of seed proteins are being exploited by inserting the sunflower albumin gene into lupin, which increases its content of sulfur-containing amino acids. White clover, which is commonly associated with bloat in cattle, has been modified by inserting a gene it lacks for the biosynthesis of tannin. It is hoped that increasing the tannin content of the leaves will reduce the incidence of bloat.

In connection with marketing of transgenic food products, Simon Bright (Zeneca Plant Science, Bracknell, UK) reported that two supermarket chains in the UK had successfully launched a tomato paste modified for better storage life. It was cheaper than its conventional counterpart, was labelled genetically modified and was well accepted.

Canola, or oil seed rape (*Brassica napus*), modified for herbicide resistance is now grown in Canada commercially. With the introduction of thioesterases and condensing enzymes from other plants, the chain length of the fatty acids in canola can

be controlled from C_8 to C_{24}, whereas the parent plant produces predominantly unsaturated C_{18} fatty acids. This, along with control of the degree of saturation, will allow oils to be tailored for particular food or industrial purposes.

Wheat flour for different food requires different properties. By controlling the positioning of cysteine residues in the glutenin in wheat, cross-linking of the protein to the amylose chains can be changed; this modifies the properties of the flour. Similarly, research into potato starch has enabled modifications that improve its use in processed foods.

In his presentation on the risks associated with outcrossing from transgenic crops, Phillip Dale (John Innes Centre, Colney, Norwich, UK) reported on the movement of genes via pollen from transgenic potatoes. The transgene was not detected beyond 10 m. In the case of canola, however, Basta resistance genes were transferred to 5% of non-transgenic canola within 50 m. From 50 to 200 m, three seeds per million were transgenic, while at 200 and 400 m there were 150 and 50 transgenic seeds per million, respectively. This work shows that with certain crops, such as potatoes, the risk of outcrossing via pollen is very low indeed. On the other hand, canola pollen can transfer to other canola plants, or near *Brassica* relatives. Fortunately, transfer to related weeds, such as wild radish, is rare, but it does occur. Consequently, when transgenic plants are grown on a large scale, genes may transfer to serious weeds. Careful monitoring of weed populations and total weed management strategies must be developed to minimize this risk.

Gene therapy

In discussions of gene therapy for cancer, it appears that it will be many years before this technology is accepted as a reliable therapeutic tool. This is evident from the variety of different approaches being tried, all aiming to inhibit tumour growth; for example, antisense strategies against oncogenes or receptors, replacing defective tumour suppressor genes, incorporating 'suicide' genes into tumour cells to increase their susceptibility to drugs and introducing immunologically significant genes (cytokines) into the tumour to invoke antitumour responses.

Systems to deliver genes for human therapy are being actively studied. Retroviral vectors to deliver genes or chemotherapeutic agents have been used in the treatment of cancers, genetic defects such as adenosine deaminase (ADA) deficiency, and HIV infection. Adeno-associated virus (AAV), which is deficient in replication, has been used to deliver DNA in a clinical trial for the control of cystic fibrosis (CF). This followed trials of the AAV vector carrying the therapeutic DNA into rabbits and rhesus monkeys suffering from CF. In these animals the DNA persisted for six months, with the gene product expressed and giving improvement in the clinical condition.

Pulmonary disease (including asthma) is common in early childhood, accounting for 70% of bed days in this age group. Some of these conditions are inherited and result from a lack of surfactant proteins (SP); a lack of SP also accompanies cystic fibrosis, which is caused by a malfunctioning of the channel transporting choloride ions across the membrane. It is now possible to study SP deficiencies in a mouse model in which the genes coding for the various SP genes are deleted (so called knockout mice). Jeffrey Whitsett (Children's Hospital, Cincinnati, OH, USA) described experiments with these mice in which cDNA with precise temporal/spatial controls of the promoter/enhancer regions, which had been identified in epithelial cells, was introduced into the mice. Such studies are providing the fundamental knowledge of lung development and function that is essential to successful therapy.

Regulations

Anthony Lubiniecki (SmithKline Beecham, King of Prussia, PA, USA) reported on regulations in the pharmaceutical industry. 'The International Conference on Harmonization' (ICH) was convened by the pharmaceutical associations of Europe, Japan and North America in 1991 in an endeavour to reach a consensus on quality control (safety, efficacy, stability, methodology), which should apply to pharmaceutical proteins made by genetic constructs. In late 1995, a consensus document was produced and is now legally binding on member countries. Although the protocols cannot guarantee freedom from risks, they offer an agreed way of minimizing them. They set out methodology for assessing the presence of virus in tissue cultures, mutations in protein products, the presence of unwanted proteins and procedures for tests on all batches post-harvesting before release.

The ICH was conscious of regulations applying in various countries and the ICH protocols are designed to be consistent with these, without pre-empting surveillance in the various member countries. The OECD has developed guidelines on the release of live genetically modified organisms (GMOs) to the open environment but these are not legally binding on OECD members. With respect to GMOs as or in food, the USA's position is that if a food can be shown to be substantially equivalent to its conventional counterpart, then it does not require labelling. However, marketing experience has shown that, at least for modified tomatoes, a label does not prejudice sales. The issue of marketing GMOs, which are sold as bulk commodities on the international market, remains unresolved.

Postscript

If one message can be distilled from the wide-ranging material presented at this '10th International Biotechnology Symposium', it is the pivotal importance of molecular biology. Long gone are the days of hitting the parent organism with a mutagen and hunting in hope of improved yield. Genes can now be precisely targeted, the DNA associated with suppressors, promoters and signal peptides can be identifed and excised or introduced to control the expression or secretion of products. Even aspects of process control, downstream processing and quality control benefit from molecular biology with the development of precise enzyme sensors for use in reactors, affinity columns for separating products and molecular probes for testing the purity of nucleic acid and protein products. The next international symposium, to be held in Brussels, will undoubtedly see further applications in this rapidly expanding industry.

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Overcoming obstacles to monoclonal antibody product development and approval

Kathryn E. Stein

Using current monoclonal antibody technology one can now produce a humanized antibody to virtually any target antigen that can be identified. Consequently, one would expect there to be more approved monoclonal antibody products. Inadequate product development at both the preclinical and clinical stages has contributed to the overall lack of success. This article discusses some of the obstacles to successful product development and offers suggestions to overcoming them. The key to monoclonal antibody development, as with other biological products, is understanding the properties of the product itself, to have some proof of concept before embarking on clinical studies, and to adequately design and power the pivotal trial.

Monoclonal antibody (mAb) technology was described by Köhler and Milstein[1] over 20 years ago. Given the exquisite specificity of mAbs and their ease of production and purification, expectations were very high for this type of biotechnology product. However, to date, there are only seven mAb products licensed for *in vivo* human use in the USA. Why have there not been more successful mAb products? There are three main reasons: (1) insufficient characterization of the product and its performance *in vitro*; (2) inadequate preclinical testing; and (3) unrealistic expectations of clinical performance, leading to inadequately designed clinical trials. This article provides examples of typical obstacles that have impeded successful product development and discusses what can be done to overcome them. For a comprehensive guide to mAb product development refer to the 'Points to consider in the manufacture and testing of monoclonal antibodies for human use (1994)' (PTC) (Ref. 2).

Product manufacturing and performance

Inadequate characterization of the specificity of the antibody, its function or its intended mechanism of action are the most common obstacles to successful product development. While it seems obvious that determining the epitope specificity of a mAb should be done before extensive product development, this has not always been the case. There have been instances when the antibody specificity was not determined until phase 3 clinical studies were well under way, or even completed. It is extremely difficult to adequately design either preclinical or clinical studies without knowing the binding specificity of the mAb and its mechanism of action. Because a large fraction of antibodies are multireactive, the sound characterization of a mAb should start with the initial (or following several rounds of antigen selection, as appropriate) screening of the fusion or library by employing appropriate control antigens. In characterizing the selected antibody it is also advisable to determine the precise epitope to which the mAb binds and to determine if the mAb reacts with related antigens. The cellular localization of the epitope and its accessibility to antibodies under conditions of the intended use of the mAb can greatly affect the potential usefulness of the mAb *in vivo* and should be carefully evaluated. In addition, as outlined in Ref. 2, mAb should be screened histologically with a panel of human tissues from several donors to determine sites of predicted or unexpected binding. If determination of the epitope specificity is delayed too long or never undertaken, the preclinical studies to determine the rationale for use, the basis for dosing and measurement of the potency of the product may prove to be inadequate for successful clinical development of the mAb.

In general, manufacturing issues have not been an obstacle to successful product development; however, inadequate studies of virus removal or inactivation have resulted in clinical studies being delayed. A lack of understanding of what was required or, in some situations, an attempt to do less than the required studies has resulted in product safety being too poorly documented to allow a study to proceed. Over the past few

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PII: S0167-7799(96)10075-5

years, validation requirements for virus removal have been modified so that early, proof-of-concept studies can be rapidly carried out for products targeted to patients with serious or life-threatening diseases for whom there are no effective alternative therapies, without compromising the safety of the patients and the general public. This approach allows antibodies to be more rapidly evaluated in these patients, at an early stage in product development and with less expenditure on validating a mAb that may not be developed beyond phase 1. Full validation studies always will be required before pivotal clinical trials. In all other situations, downstream processing for mAb produced in eukaryotic cells of animal origin should be validated for virus removal and/or inactivation, using at least one appropriate model virus, prior to use of the product in phase 1 studies. The mAb PTC outlines a new policy on generic/modular validation for use with multiple antibodies that use an identical purification scheme. This will be especially useful for manufacturers that have several mAbs of the same species and isotype, e.g. murine IgG1 antibodies, or different humanized antibodies using the same human heavy and light chain framework and constant regions, each of which is purified using the same processes. The PTC and the FDA should be consulted before implementing generic/modular validation.

Another stage at which product development has been delayed is at scale-up in manufacturing. Given the cost of scale-up, it is understandable that a sponsor would want to have prior indication of a product's effectiveness before scaling-up production, particularly when this involves constructing a new facility. In some instances, the closing of contract manufacturing facilities has resulted in the need for the sponsor to find a new contractor(s) or to construct a facility, even after the completion of a phase 3 trial. These manufacturing changes require revalidation studies of the manufacturing process and a demonstration of product comparability[3], but do not usually require a new pivotal clinical trial. In general, regulatory requirements are more easily accommodated if major scale-up is carried out prior to the initiation of a Phase 3 clinical trial but it is recognized that this is not always possible.

Preclinical development

Once specificity of a mAb product has been characterized, it is important to develop a sound rationale for use before proceeding to clinical trials. In approaching a preclinical program to develop an animal model for a mAb, two questions must be considered at the outset. First, is the target antigen present in the species under consideration for study and is it distributed in a manner similar to humans? Second, is the mAb itself a foreign protein in the species under consideration? For naked mAb, studies in animals lacking the target antigen are of little value because they have no intrinsic toxicity. In contrast, antibodies that are labeled with radionuclides, toxins, drugs or other bioactive compounds can and should be studied for toxicity in animals, even in the absence of the target antigen. Regarding the second question, the production by the host of antibodies to the mAb can complicate the interpretation of both efficacy and toxicity studies and must be taken into account. In some instances, appropriate *in vitro* studies may provide the necessary functional data to proceed with clinical development and animal studies may serve a greater role in the evaluation of dosing and toxicity.

Whenever possible, animal models should be developed that reflect the clinical setting in which the product is to be studied, and that can provide meaningful data on the biodistribution and toxicity of the product. In some instances, development of a parallel model in which the mAb is directed at the antigen of the species that the testing will be performed upon, rather than the human antigen, will provide the most relevant data. It cannot be overemphasized that the more thoroughly a product is understood before it is studied in humans, the greater the likelihood of successful clinical development. Poor preclinical development is probably the single most important contributor to failure in mAb product development in recent years. In most cases of mAb failure, had more thorough preclinical investigations been conducted the products probably would not have been studied in phase 3 trials or would have been evaluated in very different clinical trials.

Clinical trial issues

Clinical trials of mAbs have failed for a number of reasons, none of which are unique to mAb products. In mAb development, a major problem has been the rush to get products into phase 3 pivotal trials before the mAb was adequately characterized either in preclinical studies or in phase 1/2 studies. Appropriate animal models were not developed or, if animal studies were performed, the results may have been ignored in favor of 'more relevant' human studies. If the function of the product is not well understood it is difficult, if not impossible, to design an appropriate clinical trial. In some situations, a lack of knowledge about the disease being studied or changes in the practice of medicine may have compounded the problems of poor preclinical development. Early studies are essential to determine the dose of mAb and the optimal regimen for administration, to examine possible trial endpoints and to define the target and the size of the population for the study.

Finally, clinical trials often have been stopped too early. The result is that the outcome was frequently adequate for generating a hypothesis but insufficient to support the safety and efficacy of the product. A thorough understanding of the magnitude of the biological effect and the desired significance is necessary for the proper design of a pivotal trial. Another influence on the adequacy of the trial size is reliance on historical data, either for the sample size calculations or for the control group owing to changes in medical care. Even with a placebo-controlled trial, advances in the practice of medicine have resulted in lower event rates in the control group than anticipated and a *de facto*

Box 1. How to obtain FDA documents

FDA documents can be obtained as follows:
- The CBER FAX Information System, toll-free in the United States, at +1-888-CBER-FAX, or at +1-301-827-3844.
- Via the World Wide Web at 'http://www.fda.gov/cber/cberftp.html' or via the File Transfer Protocol at 'ftp://ftp.fda.gov/CBER' [connect to the FDA FTP server, FTP.FDA.GOV (192.73.61.21)]. The CBER documents are maintained in the CBER subdirectory. Logins with the user name of anonymous are permitted, and the user's e-mail address should be sent as the password. The 'READ.ME' file in that subdirectory describes the documents that may be available as an ASCII text file (*.TXT), or a WordPerfect 5.2 or 6.x document (*.w51, wp6) or both.
- By 'bounce-back e-mail'. A list of documents available by e-mail can be obtained by sending a message to DOC_LIST@A1.CBER. FDA.GOV. For each document, send a message to documentname@A1. CBER.FDA.GOV. Documents not available by return e-mail may also be requested from CBER_INFO@A1.CBER.FDA.GOV.
- Written requests may be submitted with a self-addressed adhesive label to the Manufacturers Assistance Staff (HFM-42), Center for Biologics Evaluation and Research, Food and Drug Administration, 1401 Rockville Pike, Rockville, MD 20852-1448, USA.

under-powered study. Although such changes are difficult to control, they must be taken into account when designing a pivotal trial and factored into the overall design and sample size calculations.

Conclusions

While no assurances for success of a mAb can be provided, mAbs are in general safe and potentially powerful medical tools. Success in the clinic can be maximized by knowing the epitope specificity of the antibody, being able to demonstrate its function in appropriate tests *in vitro* and/or *in vivo* and designing a phase 3 trial with adequate power, realistic expectations and clinically meaningful endpoints. Early consultation with the appropriate regulatory authorities on the programs for manufacture and clinical development can help to address specific concerns that may arise and can significantly shorten the time to approval (Box 1).

Acknowledgements

I would like to thank my colleagues, Drs E. Bonvini, M. Brunswick, M. Green, L. Miele, R. Nordan and K. Weiss for critical reading of the manuscript.

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Using phospholipases for phospholipid modification

Paola D'Arrigo and Stefano Servi

Phospholipases, which modify phospholipids in natural membranes, are available from different sources for application in biocatalysis. All sites of phospholipids can be selectively modified by enzymatic catalysis. By coupling the phospholipase action with well-established chemical transformations, natural and unnatural phospholipids can be prepared. Phospholipids find application as pharmaceuticals, food additives, cosmetics, in liposome technology and in gene transfer therapy.

Phospholipids are amphiphilic molecules that are ubiquitous in nature. They are the basic components of natural membranes and cell walls[1], where they play a structural role, and function as cofactors and activators of several membrane-associated enzymes. Hydrolysis of these compounds *in vivo* with the concomitant release of biologically active molecules (lipid second messengers: DG, IPs and PtdOH; see Glossary) is regulated by a number of enzymes (phospholipases) whose effective physiological role is still the subject of intensive research and debate. Enzymes with catalytic capacities, similar to cell phospholipases from mammals and plants, are also found in bacteria and thus provide easy access to specific enzymes for application in biocatalysis. PLs, substrates or starting materials, can be obtained as mixtures of products with different polar

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PII: S0167-7799(97)01012-3

Glossary

DAG	Diacylglycerol
IcP	Inositol 1,2-cyclic-phosphate
IP	Inositol 1-phosphate
LysoPtdCho	Lysophosphatidylcholine
PI-PLC	Phosphatidylinositol specific phospholipase C
PL	Phospholipid
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PtdAsc	Phosphatidyl-L-ascorbic acid
PtdCho	Phosphatidylcholine
PtdEt	Phosphatidylethanol
PtdEtn	Phosphatidylethanolamine
PtdEtnPeg	PEGylated phosphatidylethanolamine
PtdGro	Phosphatidylglycerol
PtdIns	Phosphatidylinositol
PtdNuc	Phosphatidyl nucleotide
PtdOH	Phosphatidic acid
PtdSer	Phosphatidylserine
PtdSial	Phosphatidylethylenedioxysialic acid
PUFA	Polyunsaturated fatty acids
PX	Generic polar head modified phospholipid

heads and acyl chains from soy beans, many vegetable oils, egg yolk, biomass and other sources. Owing to overproduction of seed oil, these can be considered as economically convenient raw materials for further transformations. Partial purification of PLs is possible by solvent partitioning and, eventually, chromatography[2,3]. In this way PtdOH, PtdCho, PtdEtn and PtdIns of different degrees of purity are available. The acyl chain composition of these compounds depends on their origin. (The fatty acid chain composition of PtdCho from soy beans is palmitic, 11.6; stearic, 3.4; oleic, 4.6; linoleic, 66.4; and linolenic 8.7%.) They are, therefore, non-homogeneous materials with mainly saturated acyl chains at the *sn*1-position and unsaturated acyl chains at the *sn*2-position (Fig. 1). Compounds of this type find applications as pharmaceuticals, food additives and in cosmetic formulations[4].

Synthetic vs biocatalytic approach

The peculiarity of this type of PL is in the nature of the polar head: less abundant PLs, such as PtdSer and PtdGro, can therefore be prepared by transformation of the more abundant components of natural PLs, i.e. PtdCho. Applications of PLs where the performance is related to phase transition properties require that compounds be chemically defined both in the acyl chain composition and in the polar head. For some applications the preparation of PLs with specific polar heads and different and defined acyl chains at the *sn*1- and *sn*2-positions is required. The preparation of this type of PL is challenging and can be effected either by total synthesis from appropriate homochiral precursors[5] or by a combination of chemical and enzymatic transformations starting from materials derived from natural PLs. There are a variety of synthetic methods that allow the total synthesis of phospholipids. For instance, a chiral glycerol derivative, protected at one of the primary hydroxyl groups, can be acylated under mild conditions to give a diacyl glycerol[6] (Fig. 2). The selective acylation occurs first on the primary alcoholic functionality, allowing the derivatization of the secondary one with a different acyl chain. After removal of the protecting group, chain migration in the diacyl glycerol thus obtained can be avoided, even when operating under mild conditions and with short reaction times. The compound is then esterified with an appropriate phosphoric ester equivalent to give an intermediate that can successively be transformed into a PL with the required polar head, shown in Fig. 1 as choline. PLs are often waxes, which cannot be easily crystallized. Compounds obtained in this way must be extensively purified by chromatography. Purity standards required for phospholipids to be used as drugs or food additives are very high. If the same compound is prepared through the biotransformation of a natural mixture of PLs, which already are generally recognized as safe (GRAS) products, the final preparation does not require purification because any impurity will be natural products. For instance, PtdSer can be prepared from PtdCho and serine by transphosphatidylation, catalysed by PLD, in up to 90% purity. Impurities can consist of PtdOH and PtdCho. This mixture does not require purification for use as a food additive, unless a compound of a higher degree of purity is required for some special purpose other than food compatibility. Thus, the enzymatic transformation of PLs has a clear advantage over their chemical synthesis. Enzymatic modifications, eventually combined with chemical steps, allow the preparation of a wide variety of innovative phospholipids of potential use in different fields. There is in fact a large patent literature, containing claims of diverse properties associated with new phospholipids that still await a practical synthesis and industrial application[7–9].

Enzymatic modifications

PLs have two carboxylic ester bonds and two phosphate ester bonds. Selective recognition requires regio- and chemoselective properties. Stereoselectivity is not necessary as the vast majority of PLs belong to the *sn*-3 family. Each of the four major phospholipases selectively recognize one of the four ester bonds (Fig. 1). Other enzymes can recognize the ester bonds in partially hydrolysed PLs (lysophospholipids); these enzymes are less common and are not useful for biocatalytic applications. In addition, non-specific enzymes with broad substrate specificity can be used in the transformation of natural PLs: ester hydrolases and phosphatases. Detailed studies of the structure, mechanism and kinetics of phospholipases have been reviewed[10,11]. All the phospholipases with potential applications are hydrolytic enzymes that are often secreted outside the cell. In principle they can catalyse both the hydrolysis and the reverse reaction, i.e. the ester bond formation in the presence of a suitable acceptor or donor. If transesterification is possible, this

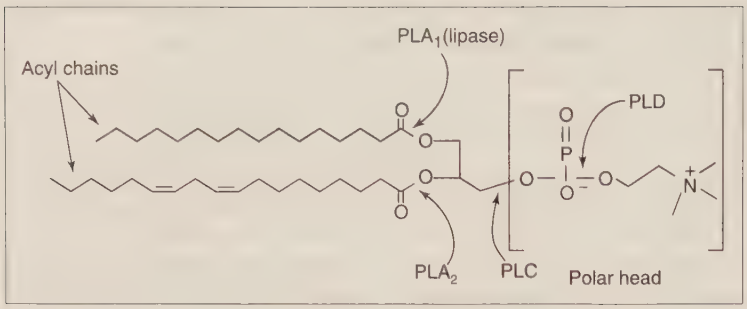

Figure 1
Phospholipases acting on PtdCho and their site of action (1-palmitoyl-2-linoleoylphosphatidylcholine shown). There is a specific enzyme catalysing the selective hydrolysis of each functionality.

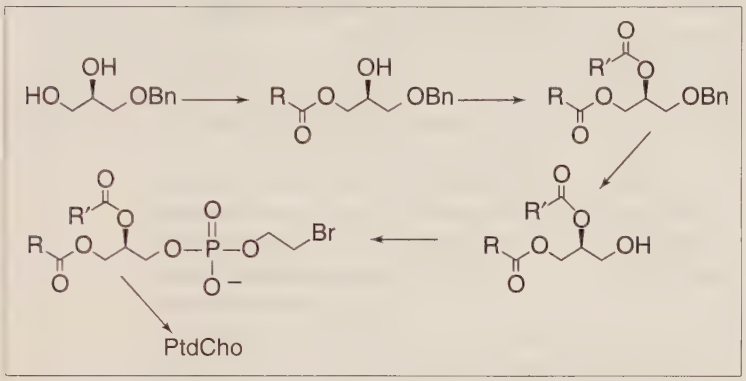

Figure 2
Synthetic pathway to PtdCho with defined acyl chains in the 2-position, starting from a chiral glycerol derivative. Reagents are not shown (see Ref. 6) but the complexity of the process is evident.

has obvious advantages over a two-step, hydrolysis–re-esterification reaction sequence, when the exchange of a particular acyl chain or polar head for another is required. Here, we describe the use of enzymes that can transform phospholipids in a synthetically useful manner.

Modification of the acyl chain at the *sn*1-position

The enzyme that has the specific capacity to catalyse the hydrolysis of the acyl chain at the *sn*-1 position is defined as PLA_1 (phosphatidylcholine 1-acylhydrolase); this enzyme is not commercially available. The same activity is present in a number of ester hydrolases (lipases) of different origin[12–14]. As these enzymes are much more accessible than PLA_1, they provide a useful alternative. *sn*1-lyso-PtdCho can be obtained by hydrolysis in the presence of one of the lipases that shows the appropriate selectivity[15]. Using water-saturated hexane and lipozyme IM20 (*Rhizomucor miehei*) from NOVO Nordisk (Denmark), up to 80% purity of *sn*1-lysoPC was obtained[16]. Almost quantitative yields of *sn*1-lysoPC and PE were obtained by hydrolysis of the corresponding PLs in a mixed-micelle system catalysed by lipase from *Mucor javanicus*[17] (Fig. 3, step a). In practical transformations, the use of mixed micelles should be avoided because product recovery becomes difficult. The products obtained in this way can be reacylated chemically with the desired acyl chain (Fig. 3, step b). Care must be taken to prevent migration of the acyl chain from position *sn*2 to the thermodynamically more stable *sn*1 position. Rapid manipulation at low temperatures in the absence of strong acids is usually sufficient to give homogeneous products. Direct transesterification of PtdCho using a carboxylic acid as the acyl donor, in organic solvent, is catalysed by the immobilized lipase from *Rhizopus arrhizus*. Incorporation of heptadecanoic acid is highly regiospecific, giving a modified PtdCho (Fig. 3, step c) in a maximum of 60% yield with controlled water activity of 0.064 (Ref. 18). In this case, separation of educt and product is, however, not possible. The lipase of this microorganism has been used previously in the biotransformation of PLs with the same selectivity[19]. Polyunsaturated fatty acids (PUFA) are recommended in diets for the partial substitution of other fatty components. The uptake of these components from PLs has been demonstrated to be more rapid than from PUFA-containing triacylglycerols. The incorporation of PUFA in PLs can be performed via transesterification with a lipase, using fish oil (high PUFA-containing triacylglycerol content) as acyl donors for the transesterification reaction. Significant levels of incorporation at both the *sn*2 and *sn*1 position of PtdCho have been observed[20].

A procedure for the preparation of alkylacylglycerophospholipids using chemoenzymatic methods is of general applicability[21]. Lipase from *Rhizopus arrhizus* is used to hydrolyse the acyl chain in *sn*1 position of phospholipid mixtures containing plasmalogen (1-*O*-1′-alkenyl) PtdCho. The lyso derivatives obtained are separated from the plasmalogen which is sequentially hydrolysed and catalytically reduced to give 1-*O*-alkyl-lyso-PtdCho which can be transformed in other ether phospholipids via acylation and PLD-catalysed transesterification.

Modification of the acyl chain in the *sn*2-position

Phospholipase A_2 (PLA_2, phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4), hydrolyses L-α-phosphatidylcholine PtdCho, to L-α-lysophosphatidylcholine LPC and fatty acid. The enzyme for biocatalytic applications is found in invertebrates (*bee*), in the venom of snakes (*Crotalus adamanteus*, *Naja naja*, *Crotalus atrox*), in mammals (bovine pancreas, porcine pancreas)[22,23] and in microorganisms (*Streptomyces violaceoruber*, *S. cinnamomeus*, *S. griseus*)[24,25]. Use of this enzyme allows the removal/replacement of the acyl chain at position *sn*2 either via hydrolysis and subsequent chemical reesterification (Fig. 3, steps d,e) or through direct interesterification with an acyl donor (Fig. 3, step g). Hydrolysis reactions are usually more efficient than transesterifications. Quantitative removal of the acyl chain in position *sn*2 can be effected in mixed micelles[22] but biphasic systems are preferred because of the easier isolation of the product[26]. Reactions other than hydrolysis occur with limited yields.

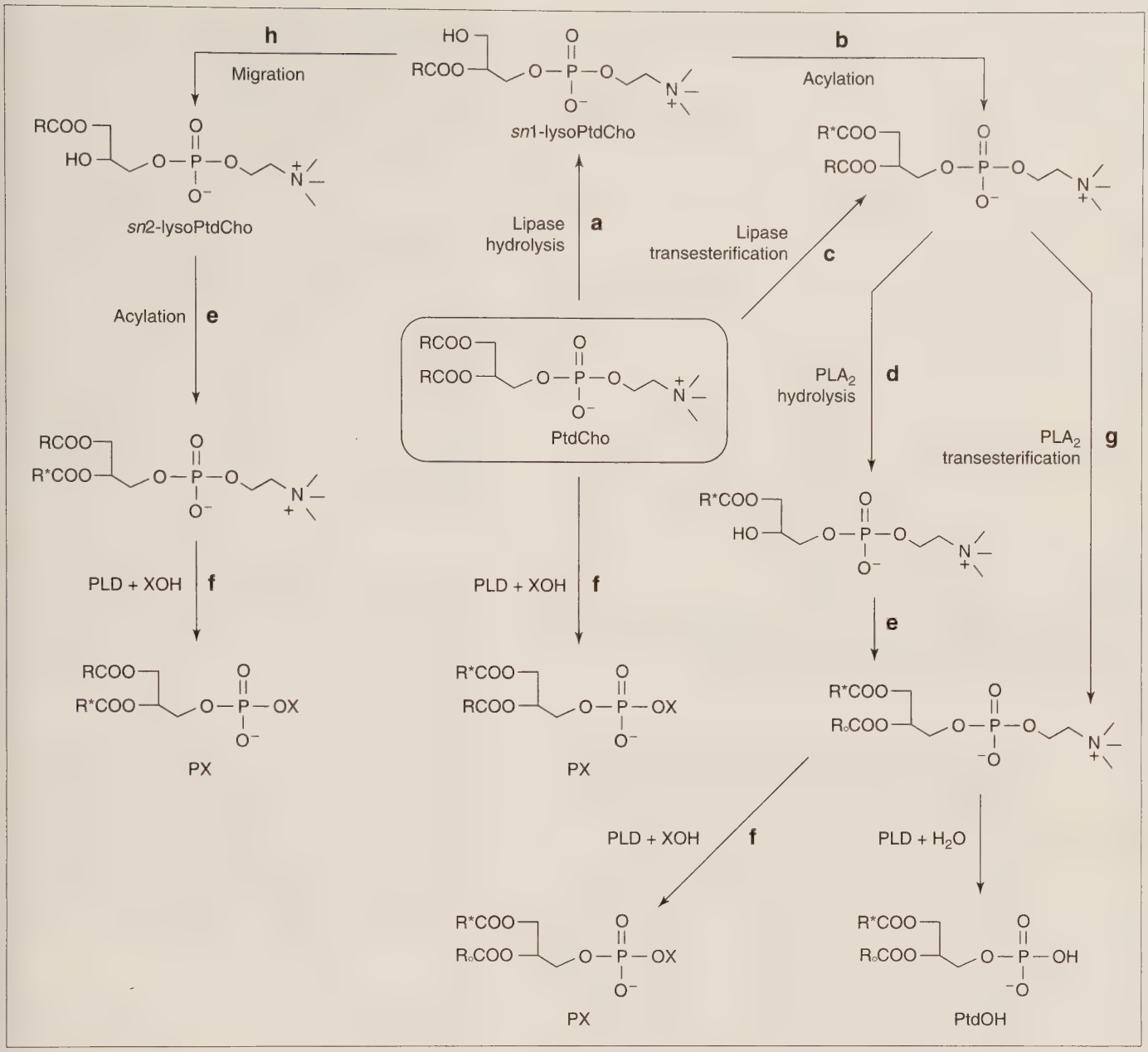

Figure 3

Modification of PtdCho by combined use of lipases, PLA_2 and PLD. Path **f** is the most useful in large-scale preparation. PtdSer, PtdEtn and PtdGro are prepared in this way.

The direct interesterification of PtdCho with myristic acid in the presence of immobilized-PLA_2 from different sources has been reported to give up to 43% incorporation of the myristoyl group at position *sn*2 (Ref. 27). Starting with *sn*2-lysoPC, modest incorporation of fatty acids has been obtained either in a biphasic system or in an organic solvent with low water content[25,26,28,29]. PLA_2 is specific for PLs with the natural absolute configuration at position *sn*2 (Ref. 30). This property could be used to advantage in the resolution of racemic PLs or in assessing the absolute configuration of PLs of different origin.

The modification of both acyl chains in PC could be achieved either via steps a, h and e (which are equivalent to steps d, e) or through steps a, b, d or c, d, e. This methodology coupled with synthetic steps has been applied to the preparation of PtdEtn, PtdOH and PtdIns with two different acyl chains[7–9]. A specific example of the preparation of a particular phospholipid is the semi-synthesis of the antifungal agent lysofungin[31]. The major component of soybean phosphatidylinositol, 1-stearoyl(palmitoyl)-2-linoleoyl-3-glycerophosphatidyl-D-*myo*-inositol, is deacylated at the *sn*1 position with a preparation of *Rhizopus arrhizus* lipase at pH 6.5 to give a homogeneous 1-lyso-PtdIns. Mild treatment with 50 mM TAPS buffer at pH 8.5 causes linoleoyl group migration, giving the desired natural product in good yield (Fig. 4).

Modification of the polar head

Modification of the polar heads of natural or synthetic phospholipids by phospholipase D is perhaps the

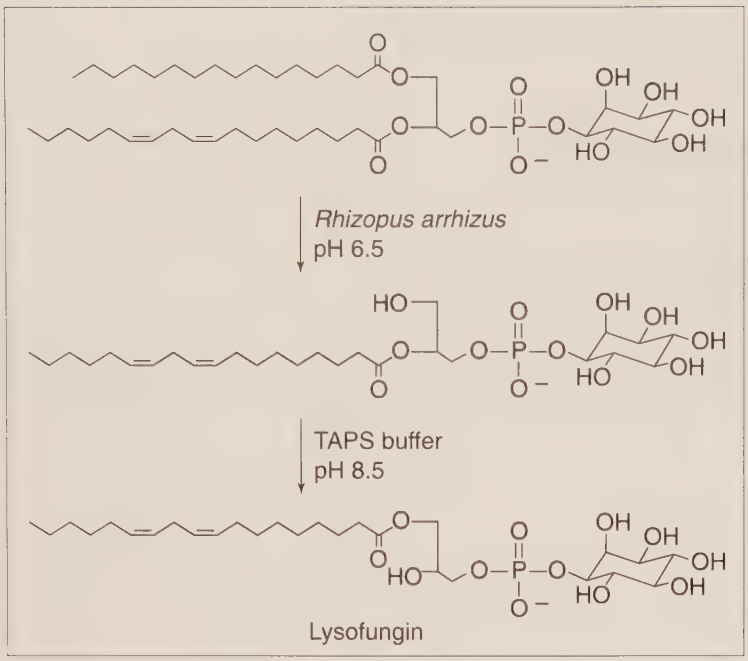

Figure 4

Preparation of 1-linoleoyl-2-lysophosphatidyl-myo-inositol via regioselective lipase hydrolysis and acyl migration. The thermodynamically more stable product is obtained in high purity. The corresponding chemical synthesis would be possible but much more complex. In this case the starting material is a natural product.

most commonly employed enzymatic modification in the preparation of modified phospholipids[32]. Phospholipase D (PLD, phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) hydrolyses L-α-phosphatidylcholine to phosphatidic acid and choline. In the presence of an alcohol, transphosphatidylation can occur with different degrees of selectivity depending on the enzymatic source, the nature of the alcohol and its concentration. Products of the reaction will be phosphatidic acid (PtdOH) and a new phospholipid modified in the polar head (PX). PLDs from different sources are commercially available. Cabbage PLD can be easily prepared from homogenates of Savoy cabbage[33]. This enzyme is effective for the preparation of PtdOH and PtdGro, but when reacted in the presence of less-reactive alcohols, low ratios of PX:PtdOH are obtained. As purification of products is not easy, this type of transformation is not synthetically useful. PLD from the culture broth of *Streptomyces* are commercially available or can be prepared by fermentation from a number of *Actinomycetes* strains[34-36]. They have a very broad substrate specificity for both partners of the reaction. Whereas PtdCho is invariably the preferred substrate, with K_m = 0.4 mM (emulsion system, hydrolysis), most natural and unnatural phospholipids are substrates for both hydrolysis and esterification. These include PtdEtn, PtdGro, PtdSer, sphyngomyelin, ether phospholipids and non-glycerophospholipids, such as long-chain fatty acid esters of phosphorylcholine. Most primary alcohols are co-substrates for the transphosphatidylation reaction. Phosphatidylethanol (PtdEt) is formed in an extremely efficient way from PtdCho and ethanol with PLDs of different sources. This reaction is used to monitor the presence of PLD in tissues (formation of PtdEt in the presence of added ethanol)[37]. Most primary alcohols, irrespective of the complexity of their structure, are phosphatidyl acceptors. Secondary alcohols are also incorporated when they are the only alcoholic components but with lower efficiency than primary alcohols[38]. Several cycloalkanols are also substrates for bacterial PLD, but inositol and its phosphates are not accepted. This is also the case for PtdIns, which is not a substrate for the hydrolysis reaction. Reactions are best performed in emulsion systems formed by a phospholipid solution in a water immiscible organic solvent and the enzymatic solution buffered at pH ~5.6. The alcohol concentration in the transesterification reaction is kept as high as possible to minimize PtdOH formation. However, high alcohol concentration can reduce the enzymatic activity by inhibition. Membrane bioreactors have been used with no apparent advantage over the conventional biphasic configuration[33].

Enrichment of phospholipid mixtures

PLD-catalysed transformations have been used in the enrichment of PtdCho in a mixture of PLs containing PtdCho and PtdEtn. Thus, treatment of the mixture with choline in a biphasic system, in the presence of bacterial PLD, results in the complete transformation of PtdEtn into PtdCho (Ref. 39).

The purification of PtdIns from the same mixture of phospholipids is also possible using a similar technique. Since PtdIns is only hydrolysed by a PtdIns-specific PLD, PtdCho and PtdEtn can be converted into PtdOH, using the enzyme from *Streptomyces* sp., leaving PtdIns unchanged. Subsequently, the PtdOH can be hydrolysed by acid phosphatase. Thus, PtdIns can be easily separated from DG owing to its strikingly different solubility in organic solvents[40].

Preparation of natural and unnatural polar head modified phospholipids

The method described for the preparation of polar head modified PLs is a general one and can be associated with other phospholipase-catalysed transformations, as shown in Fig. 2. The method has been applied in the industrial preparation of less-abundant natural PLs such as PtdEtn (Ref. 41), PtdGro (Ref. 42), PtdSer (Ref. 43) and PtdOH (Ref. 44), and for the preparation of modified ether phospholipids[45] and other special PLs (Refs 46,47; Fig. 5).

For example, PtdEtnpeg is used in the preparation of sterically stabilized liposomes[48]. Phospholipids in which the principle is linked to polar head groups can act as prodrugs with PLD-catalysed hydrolysis serving to release the drugs *in vivo*. 6-Phosphatidyl-L-ascorbic acid (PtdAsc) displays special antioxidant properties in food[49], and phosphatidyl nucleotides (PtdNuc) show promise as antitumour agents[50]. In addition, phosphatidyl sialic acid (PtdSial) has been proposed as a potential drug[51]. Inhibition of cell-surface receptors or enzymes is effectively enhanced if the monomeric inhibitor is oligomerized or clustered, therefore the

incorporation of the inhibitor into the polar head of a phospholipid via PLD-catalysed transformation is believed to be a general approach to the delivery of such inhibitor molecules[52]. Clearly, for all the compounds in Fig. 5, chemical synthesis presents more serious problems than the biocatalytic approach.

Phospholipase C

Phospholipase C (PLC, phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) hydrolyses L-α-phosphatidylcholine to diglyceride and choline phosphate. Three different common PLC enzymes from microbial sources are PLC_{PtdIns}, PLC_{PtdPC} and PLC_{SM}. The first of these is specific for PtdIns, giving DAG and inositol cyclic-phosphate (IcP) as hydrolysis products. PLC_{PtdCho} has rather broad substrate specificity, hydrolysing PtdCho and other glycerophospholipids, whereas PLC_{SM} is specific for sphyngomyelin hydrolysis[53]. Cultures of *Bacillus cereus* and *B. thuringiensis* are suitable for the production of the enzyme for biocatalytic applications. The use of the diacylglycerols, or of the phosphate esters obtained in the hydrolysis, has not found large-scale application.

The PLC-catalysed transfer reaction of a phosphorylcholine moiety has been reported[54]. In this reaction, one phosphorylcholine moiety is transferred from PtdCho to *N*-oleoyl-sphingosine with formation of sphingomyelin. This reaction is potentially of great interest, but its generality has not been further investigated. Very recently, inositol 1,2-cyclic-phosphate, obtained by PLC_{PtdIns}-catalysed hydrolysis of PtdIns from soy beans, was transformed into *O*-alkyl-inositol 1-phosphates via a transesterification reaction in the presence of primary alcohols, catalysed by the same enzyme. In this way a large number of structurally analogous inositol phosphate diesters of different complexity have been obtained on a useful preparative scale[55] (Fig. 6).

Biocatalytic approach to PLs

It appears that catalysis by hydrolytic enzymes acting on phospholipids, either in hydrolysis or in transesterification, provides routes for obtaining PLs with the natural absolute configuration at the *sn*2 centre, different acyl chains at the 2-positions and almost any polar head containing primary or secondary alcohols. The advantages over chemical synthesis are evident if the product's destination is the food or pharmaceutical industry: purification from starting materials or by-products that are harmless, accepted compounds is

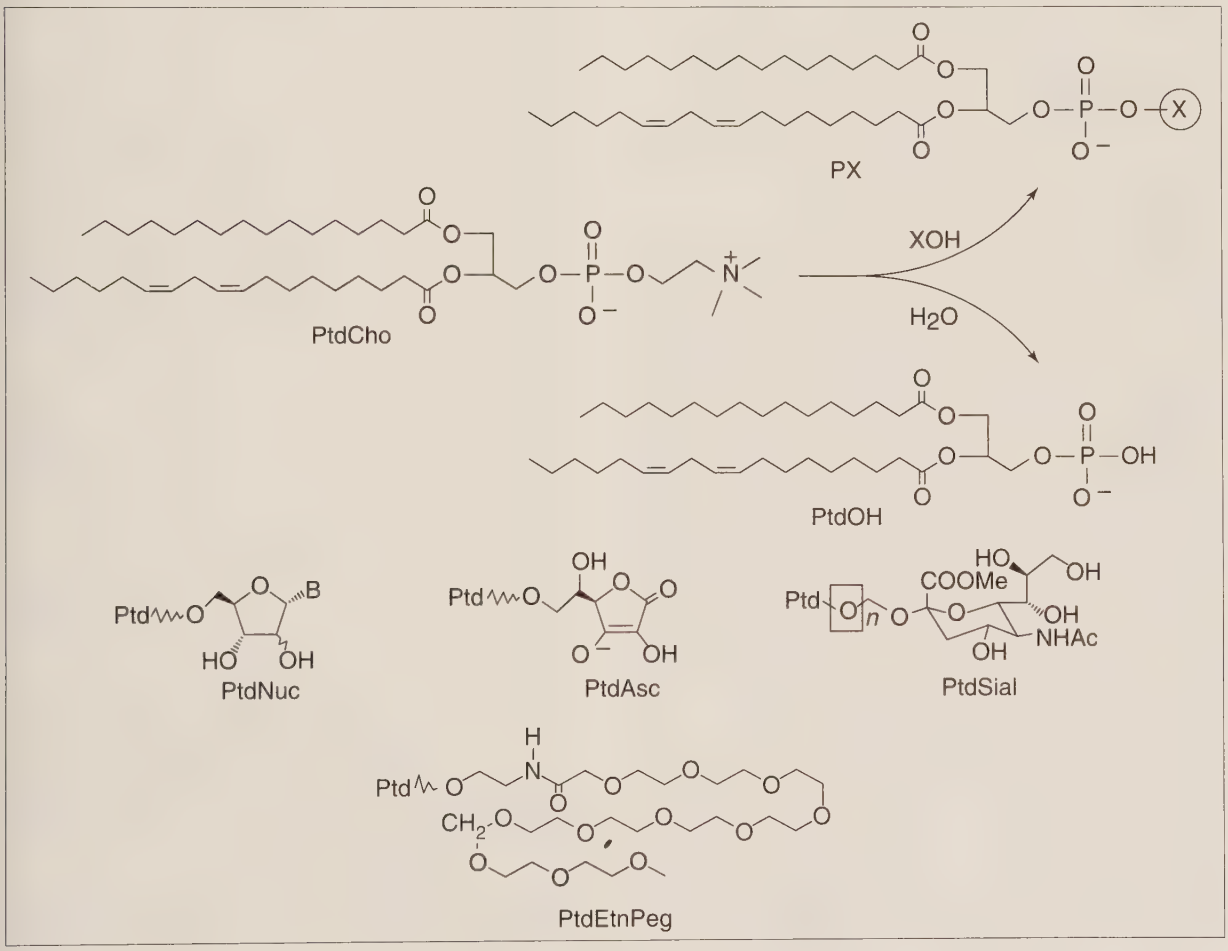

Figure 5

Synthesis of phospholipid conjugates by PLD-catalysed polar head modification of PtdCho. For use in the food and pharmaceutical industry, PtdCho and PtdOH impurities in the target PX can be tolerated. If the process occurs with high selectivity, purification can be avoided.

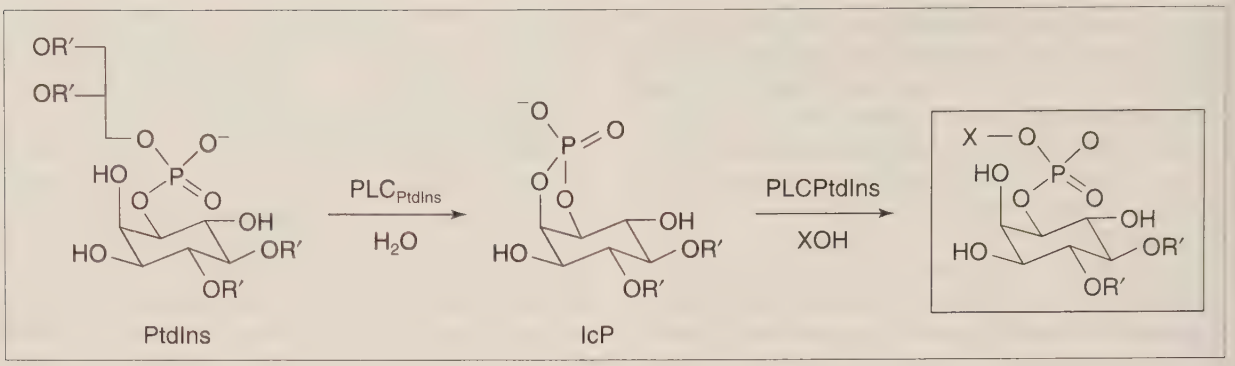

Figure 6

Preparative transesterification catalysed by PLC$_{PtdIns}$. The IcP initially formed in water from PtdIns is transformed in the presence of primary alcohols into *O*-alkyl inositol 1-phosphates of various structural complexity (R′ = H, phosphate. XOH = primary alcohol).

much safer and easier than purification from chemical reagents. There is a large number of potentially useful new phospholipids whose properties are claimed, especially in the patent literature, which still await a practical synthesis for industrial application. Preparation of these PLs from natural ones via phospholipase modification appears to offer an interesting approach to this problem.

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Why are enzymes less active in organic solvents than in water?

Alexander M. Klibanov

In order to exploit fully the biotechnological opportunities afforded by nonaqueous enzymology, the issue of often drastically diminished enzymatic activity in organic solvents compared with that in water must be addressed and resolved. Recent studies have made great strides towards elucidating causes of this phenomenon of activity loss. None of these causes is insurmountable; by designing strategies that systematically target them, enzymatic activity in organic solvents can be readily enhanced by multiple orders of magnitude and ultimately brought to the aqueous-like level.

Over the past decade, nonaqueous enzymology has emerged as a major area of biotechnology research and development[1]. Although the notion of enzymatic processes in organic solvents was initially met with scepticism by the scientific community, nonaqueous enzymology is now studied and exploited by numerous academic and industrial laboratories worldwide, and the number of papers devoted to this topic has surged into the hundreds[1].

The reason why nonaqueous enzymology has generated so much excitement is that enzymes exhibit striking new properties in organic solvents. For example, in organic solvents enzymes often catalyze reactions impossible in water and are very stable[1]. Furthermore, enzyme selectivity in organic solvents is not only distinct from that in water but can be markedly controlled, and even reversed, by the solvent[2,3]; this 'solvent engineering' thus provides an alternative to protein engineering.

A number of applications for enzymes in organic solvents have been developed in chemical processing (particularly for the synthesis of optically active intermediates), food-related conversions and analyses[1]. The only dark cloud hanging over nonaqueous enzymology is the notion (largely, albeit not always, correct) that enzymes in organic solvents are far less active than in water. For instance, the proteases α-chymotrypsin and subtilisin have activities 10^4–10^5-times lower in anhydrous octane than in water; the two enzymes are less active still in most other organic solvents[4]. Such numbers seem to be typical, although two comments must be made. First, while handicapped by transfer from water to organic solvents, enzymes nevertheless remain impressive catalysts in organic solvents. For example, in the case of α-chymotrypsin and subtilisin, the specificity constant, k_{cat}/K_M, of an enzymatic transesterification reaction in anhydrous octane exceeds the bimolecular rate constant of the same reaction without enzyme by as much as 10^{11}-fold[4]. Second, whereas the rates of enzymatic processes in anhydrous solvents are indeed dwarfed by those in water, this is not necessarily so in hydrous ones. For example, polyphenol oxidase in octanol containing 3% water retains more than a third of its activity in water[5]. Likewise, the activity of yeast alcohol dehydrogenase in isopropyl ether containing 0.5% water is about a quarter of that in aqueous solution[5].

These comments notwithstanding, the fact that enzymatic activity in anhydrous or nearly anhydrous solvents falls short of that in water clearly threatens the practical potential of nonaqueous enzymology (although it should be kept in mind that from the industrial standpoint the real question is whether the enzymatic activity is high enough for the intended application, not how it compares with that in water). Therefore, the issue of activity loss must be confronted and, hopefully, alleviated. The only way to do so rationally is to elucidate why enzymatic activity plummets on transition from water to anhydrous solvents. This elucidation, much advanced by recent studies, is the subject of the present article. The discussion will involve, primarily, the model enzyme subtilisin Carlsberg, for which the bulk of the relevant work has been done and the greatest insights have been attained. Two enzyme forms introduced into organic solvents will be considered: amorphous (e.g. lyophilized, which is the most popular form among researchers[1–5]) and crosslinked crystalline (CLC) enzymes[6].

Diffusion and accessibility factors

Enzymes are soluble in water but insoluble in nearly all organic solvents. Hence, the addition of enzymes

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PII: S0167-7799(97)01013-5

leads to solutions in water but, usually, suspensions in organic solvents. Consequently, it is intuitively plausible that enzymatic activity in nonaqueous solvents may be diminished due to diffusional limitations on the substrates. This phenomenon, common in heterogeneous, including immobilized enzyme, catalysis[7], results in the under-utilization of enzymatic power and thus decreased observed activity. Theoretical analysis[8] shows that such mass transfer limitations may indeed slow down enzymatic catalysis in organic solvents. We experimentally tested this possibility in the specific case of subtilisin. If subtilisin catalysis in organic solvents is suppressed by diffusional limitations, then the dependence of the enzymatic activity in such media, or that in water, should be nonlinear (and should eventually level off); by contrast, a linear dependence should ensue in the absence of diffusional restrictions[7,9]. In fact, we observed linear dependences in all three organic solvents examined: dodecane, carbon tetrachloride and acetonitrile[9]. Therefore, the 5000–100 000 times slower catalysis in these solvents compared with that in water[4] cannot be caused by mass transfer limitation.

Even in the absence of diffusional limitations *per se*, it is conceivable that in a lyophilized enzyme particle or CLC enzyme, some enzymatic active centers may be sterically shielded by the neighboring enzyme molecules and thus be inaccessible to the substrate. This would effectively prevent those enzyme molecules from participating in catalysis, thereby cutting the observed catalytic activity. This possibility was tested by titrating the competent active centers in subtilisin and α-chymotrypsin suspended in organic solvents. The data obtained[4,10] reveal that in octane the number of centers is between one- and two-thirds of that in water. For CLC subtilisin in organic solvents it is even higher – essentially unity[11]. Therefore, steric blockage can be responsible for only a tiny fraction of the loss of enzymatic activity on transition from water to organic solvents.

Structural changes

Another obvious suspect among the possible causes of the diminished enzymatic activity in anhydrous solvents compared with that in water is a change in enzyme conformation. Such protein denaturation in aqueous–organic mixtures is well known[12], and thus it would not be unreasonable to expect an even graver situation in neat organic solvents. For CLC enzymes suspended in anhydrous solvents, this issue can be addressed experimentally by means of X-ray crystallography. Several years ago we succeeded in solving the crystal structure of CLC subtilisin in anhydrous acetonitrile[13]. Comparison of this structure with its counterpart in water[13,14] revealed that the two are virtually identical. The same holds true for the recently solved crystal structure of CLC subtilisin in dioxane (see the cover of this journal issue). Likewise, the X-ray crystal structure of uncrosslinked chymotrypsin in hexane was found to be very similar to that in water[15]. Therefore, factors other than conformational change must underlie, for example, the 10^7-fold difference in enzymatic activity (k_{cat}/K_M) between subtilisin dissolved in water and CLC subtilisin suspended in acetonitrile[11].

X-ray crystallography, unfortunately, cannot be applied to lyophilized enzymes suspended in organic solvents, thus making complete structure characterization of such systems impossible. Nevertheless, some structural information can be derived by other biophysical methods, such as Fourier-transform infrared (FTIR) spectroscopy. Using this methodology we have recently ascertained that placing lyophilized subtilisin in a variety of anhydrous solvents, such as octane, acetonitrile and dioxane, has no appreciable effect on its secondary structure (as reflected by the α-helix content)[16]. However, the preceding step, lyophilization, does result in a significant (and reversible if lyophilization is done carefully) denaturation of subtilisin[16] and many other proteins[17,18]. In other words, somewhat ironically, it is not the contact with an organic solvent but the prior dehydration that alters the enzyme structure and, undoubtedly, lowers catalytic activity. This deleterious effect can be prevented, or at least minimized, by lyoprotectants, as discussed in a later section.

Energetics of substrate desolvation and transition state stabilization

The energy of binding between the enzyme and the substrate is the major driving force for enzymatic catalysis and the source of huge rate enhancements afforded by enzymes[19]. In order for binding to occur, the substrate must first undergo desolvation upon its journey from the reaction medium to the enzyme active center. The more energetically favorable this desolvation is, the greater the net binding energy (resulting in catalysis) becomes. Subtilisin, α-chymotrypsin and many other enzymes have hydrophobic active centers; they work best with hydrophobic substrates because there is a large energetic incentive for the latter to partition from water into the active center. When water is replaced with an organic solvent, the substrate is no longer 'squeezed out' of the medium owing to the hydrophobic effect and the energetic advantages of the partitioning drop – or, in thermodynamic language, the ground state of a hydrophobic substrate is stabilized in organic solvents relative to water[20]. This ought to raise the activation barrier and hence slow down the enzymatic reaction[19].

Using CLC subtilisin (where, as discussed above, there are no conformational changes upon replacement of water with anhydrous reaction media), we have quantified the decline in enzymatic activity stemming from this less favorable substrate desolvation. In the case of the substrate N-Ac-L-Phe-OEt, this effect accounts for more than a 100-fold reduction in k_{cat}/K_M in anhydrous acetonitrile compared with that in water[11].

Another term affecting the activation energy (and thus enzymatic reactivity) is the energy of the transition state. For subtilisin and many other hydrolases, this

reaction transition state is highly polar (resembling a charged tetrahedral intermediate)[19]. If it is fully enveloped by the enzyme active center (i.e. shielded from the solvent), a solvent change should not affect its energy. However, full shielding does not always occur – for example, at least one-third of the transition state of subtilisin with N-Ac-L-Phe-OEt is exposed to the solvent[2]. Since water stabilizes tetrahedral intermediates much better than less polar solvents, the enzymatic activity in the latter should be lower as a result of this phenomenon[21]. Enzyme–substrate interactions in partially-exposed transition states may also be affected by the solvent[20].

Conformational mobility

Interaction of proteins with water (hydration) is critical for a plethora of biological functions, particularly enzymatic activity[22]. Water, acting as a lubricant or plasticizer[23], allows enzymes to exhibit the conformational mobility required for optimal catalysis. Organic solvents, in general, are not nearly as accommodating, if accommodating at all, because they lack water's ability to engage in multiple hydrogen bonds (and, because of their lower dielectric constants, lead to stronger electrostatic interactions and hence more rigid proteins[24]). These considerations dictate that, other things being equal, enzymes should be less active in anhydrous solvents than in water owing to restricted conformational mobility. In fact, enzymes exhibit significant catalytic activity in such media only because they strongly bind some 'essential' water and retain it even when suspended in anhydrous solvents[4].

There are numerous lines of evidence supporting this analysis. There is a general inverse correlation between enzymatic activity in an organic solvent and the latter's hydrophilicity – the highest activity is usually observed in hydrophobic solvents[4,25]. This is because hydrophobic solvents possess a lesser ability than their hydrophilic counterparts to strip the essential water off enzyme molecules[4]. Addition of water to enzyme suspensions in anhydrous solvents[5] or increasing the thermodynamic activity of water (a_w) in such systems by other means[26] can dramatically enhance enzymatic activity. To some extent, water can be replaced by water-mimicking organic solvents, such as glycerol or ethylene glycol, which can also form multiple hydrogen bonds[5,27].

Note that CLC subtilisin loses almost two orders of magnitude in catalytic activity solely due to the decline in its conformational mobility in anhydrous acetonitrile compared with that in water[11].

pH situation

In aqueous solution, enzyme catalysis profoundly depends on the pH and has a pH optimum[19]. Since the notion of pH has no meaning in organic solvents, the protonation state of enzyme ionogenic groups must be controlled by other factors. It was discovered[4,25] that enzymes have a 'pH memory' – i.e. their catalytic behavior in organic solvents reflects the pH of the last

Table 1. Causes of lower enzymatic activity in organic solvents compared with that in water and ways to alleviate them[a]

Cause[b]	Comments	Remedies[b]
Diffusional limitations	Not as likely as widely claimed	Vigorously agitate enzyme suspensions; use small enzyme particles
Active center blockage	Responsible for no more than a few-fold activity reduction	If matters, use crystalline rather than amorphous enzyme particles
Conformational change	Occurs upon lyophilization and other modes of dehydration. Is avoided with CLC enzymes. Is usually not caused by contact with the solvent	Use lyoprotectants; alternatively, prepare enzyme complexes with amphiphiles soluble in organic solvents; use enzyme CLCs
Unfavorable energetics of substrate desolvation	Severe with hydrophobic substrates; also, likely with natural substrates. May be responsible for at least a 100-fold activity reduction	Select the solvent expected to yield unfavorable solvent–substrate interactions
Transition state destabilization	Likely to be pronounced when the transition state is at least partially exposed to the solvent	Select the solvent expected to yield favorable interactions with the transition state
Reduced conformational mobility	Severe in anhydrous, hydrophilic solvents owing to the stripping of the essential enzyme-bound water. May be responsible for at least a 100-fold activity reduction	Optimize water activity (a_w); hydrate the solvent; use hydrophobic solvents; use water-mimicking and denaturing cosolvent additives
Suboptimal pH situation	May be responsible for at least a 100-fold activity reduction	Dehydrate from aqueous solution of the pH optimal for enzymatic activity; use organic-phase buffers

[a]The table refers to lyophilized (or other amorphous preparations) or crosslinked crystalline (CLC) enzymes suspended in neat organic solvents.
[b]Both proven and hypothetical (see text).

aqueous solution to which they were exposed (e.g. from which they were lyophilized). Therefore, unless enzyme powders are obtained from an aqueous solution of the pH affording maximal activity, enzymatic performance in organic solvents is doomed to be suboptimal.

An alternative to recovering enzymes from aqueous solutions at appropriate pH values is to employ organic-phase buffers – i.e. suitable mixtures of organic-soluble acids and their conjugate bases[28]. This approach has been successfully used to increase markedly the catalytic activity of lyophilized[28] and CLC (Ref. 29) enzymes in organic solvents.

How to enhance enzymatic activity in organic solvents

The foregoing analysis shows that there are several physicochemical factors that may lower enzymatic activity in organic solvents compared with that in water (Table 1). Gratifyingly, virtually all examples of enzyme activation in nonaqueous media reported in the literature to date can be explained in terms of minimizing these individual factors.

The most popular strategy has been to prevent enzyme denaturation during dehydration. This has been achieved by lyophilizing enzymes in the presence of lyoprotectants such as sugars[30], poly(ethylene glycol)[30], inorganic salts (notably KCl)[31], substrate-resembling ligands[30,32], and crown ethers[33]. An alternative is to preform organic-soluble complexes of enzymes with detergent (or other amphiphile) molecules[34,35]; enzymes are apparently in native-like conformations in such complexes[16,35]. These approaches have resulted in increasing enzymatic activities in organic solvents by up to three to four orders of magnitude.

Another effective strategy has been to 'loosen up' enzymes in anhydrous solvents. Enzymatic activity has been increased up to two to three orders of magnitude by adding small quantities of water to organic solvents[5], maximizing a_w (Ref. 26), and adding water mimics[5,27] or denaturing cosolvents[36] to anhydrous media.

Concluding remarks

The reasons why enzymatic activity in organic solvents usually does not measure up to that in water have been elucidated (Table 1). In some instances, their contributions to the overall activity loss have been quantified, and the sum thereof comes close to the observed decline in the catalytic performance in nonaqueous media compared with water[11]. None of these factors is insurmountable and, as the aforementioned examples illustrate, can be prioritized, systematically tackled (Table 1) and, perhaps, eliminated. Indeed, 'aqueous-like' activities of enzymes in nonaqueous solvents have thus been achieved[5,35] and less spectacular but nonetheless quite dramatic activations are now commonplace[4,10,11,26–36]. There is every reason to be optimistic that, by judiciously combining the remedies discussed above, enzymatic activities in organic media can be routinely made comparable with those in water. In fact, it may even be possible to make enzymes more active in organic solvents than in water. For example, particularly for unnatural substrates, the energetics of substrate desolvation may be made more favorable. Also, there is the intriguing possibility of molding enzyme active centers and thus optimizing enzymatic activity in organic solvents for a desired substrate by molecular imprinting[32,37].

There is now convincing evidence dispelling the popular cliché that enzymes should be more active in water than in organic solvents because that is where Nature intended to use them. Regardless of Nature's intention, if optimally used, enzymes may be able to work as well in nonaqueous as in aqueous media. The real question thus becomes: what novel mechanistic knowledge and useful practical applications will be derived from this opportunity?

Acknowledgements

Work in the author's laboratory was supported by grants from the National Institutes of Health (GM39794) and Department of Energy (BCTR program). I am grateful to Jennifer L. Schmitke for helpful discussions.

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Atomic force microscopy in analytical biotechnology

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The past decade has seen the atomic force microscope evolve not only as a high resolution imaging tool, but also as an instrument capable of measuring forces between surfaces and the material properties of samples. Here, the use of atomic force microscopy for surface force measurement is reviewed, highlighting the considerable progress that has recently been made in the area of biotechnology. Particular emphasis is placed on how the instrument can be used to probe directly biomolecular interactions, illustrating the potential of the technique to impact on our fundamental understanding of molecular structure and processes.

Since its invention in 1986, the atomic force microscope (AFM)[1] has evolved as a valuable imaging technique with resolution in the micrometre to subnanometre range. The ability of the AFM to image both insulating and conducting surfaces in a variety of environments has facilitated molecular resolution images of a wide range of biomolecules, including proteins[2], lipids[3] and DNA (Ref. 4). In addition to its imaging abilities, the AFM can also be employed to probe spatial variations in surface properties, such as adhesiveness and elasticity[5]. The use of the AFM as an imaging technique in the study of biomolecules has frequently been the subject of review[6,7]. The aim of this article is to outline the use of an AFM as an instrument for surface force measurements, with a particular emphasis on how the technique can be employed in the areas of biotechnology and biological science.

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The AFM as a force sensing instrument

Intermolecular forces govern the fundamental properties of solids, liquids and gases, the properties of colloids and the organization of biological structures[8]. Numerous biophysical methods have been employed to investigate specific and non-specific molecular interactions, including optical trapping[9], magnetic force experiments[10], pipette suction[11] and the surface force apparatus[12]. Atomic force microscopy complements these techniques by uniquely combining an extended force range with high spatial resolution. The AFM has a theoretical force sensitivity of 10^{-15} N (Ref. 13), and employs probes with a tip apex of 10–50 nm radius[14], to produce contact areas as small as 10 nm^2; therefore providing a means to overcome the limitations of other techniques.

By recording force measurements between different probe–sample combinations in various ionic media, it was first illustrated that the AFM could be used to measure forces, such as van der Waals and electrostatic forces[15]. Ducker and colleagues[16] also employed the technique to measure colloidal forces, by attaching spherical particles to the apex of AFM cantilevers. The

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PII: S0167-7799(97)01015-9

ability of the instrument to measure discrete adhesive forces of 10 pN was first highlighted by Hoh *et al.*[17], who attributed the force to that required to rupture a single hydrogen bond. Using these applications of the instrument, various groups then began to analyse the forces controlling more complex systems, including those between polymer-coated surfaces[18,19] and protein–protein interactions[20–31].

Principle of operation

In the AFM a sharp probe is attached to a cantilever spring, which is scanned in close proximity to a sample surface. During scanning or 'imaging' the cantilever is deflected by forces acting between the surface and the probe (Fig. 1). To produce an 'image' the deflections of the cantilever are monitored with respect to its lateral position on the sample (reviewed in Refs 6,7). However, the cantilever deflections may also be used to directly measure the forces acting between the probe and sample.

When using the AFM probe–cantilever system to detect forces, the deflections of the cantilever are monitored as the probe and sample are brought into contact (approach trace) at constant velocity, and then separated (retract trace). The resultant plot of cantilever deflection versus distance moved by the piezo controlling the height of the probe or sample (depending on instrument design) is shown in Fig. 2.

As the probe–sample separation is reduced, the cantilever is deflected from its rest position by forces acting on the probe; attractive forces making the cantilever bend towards the sample and repulsive forces deflecting the probe away from the surface. Forces that can be detected in the non-contact region of the force cycle include van der Waals (attractive) and electrostatic (attractive or repulsive) forces. Once in contact with the sample surface any further forward motion produces a repulsive force, as the electron orbitals of the atoms in the probe and sample begin to overlap (Born repulsion). In addition to the bending of the cantilever in the contact region of the force curve, the probe and sample may undergo elastic (reversible) or plastic (irreversible) deformations as the load is increased.

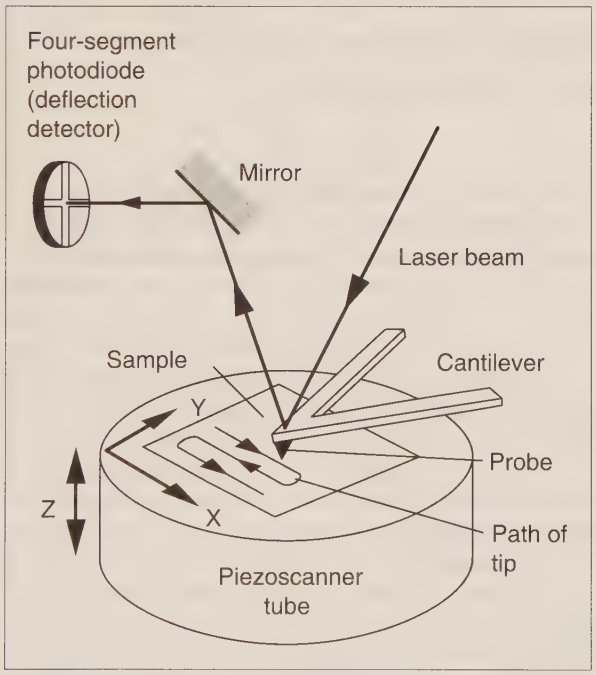

Figure 1

Contact mode imaging using an atomic force microscope. Typically, the probe is raster scanned (see path of tip) while touching the sample surface. To produce a topographic map of the sample surface, the deflections of the cantilever are monitored using an optical lever. The lateral and vertical positions of the probe (or sample depending on instrument design) during scanning are controlled using piezoceramics.

After the point of maximum applied load is reached, the direction of motion is reversed and the probe and sample are separated. During the retraction phase of the measurement the probe often sticks to the surface due to interactions between the probe and sample. As the probe–sample separation is increased further the probe is pulled away from the sample surface and the cantilever deflection returns to its original rest position.

The distance d the cantilever is deflected from its rest position is directly proportional to the force F acting between the probe and surface, as stated by Hooke's Law ($F = -kd$). The constant k in this case represents the spring constant of the cantilever spring. The spring constants of commercially available AFM cantilevers have been found to vary significantly from the value stated by the manufacturer[32]. For the accurate measurement of probe–sample interaction forces the calibration of the cantilever spring constant is, therefore, essential. The most common calibration methods rely on the addition of small tungsten beads (mass approx. 10^{-9} g) to the ends of cantilevers [see Fig. 2 (inset)]. The spring constants can then be calculated from the change in the cantilever resonant frequency on the addition of the tungsten bead[32], or from the cantilever deflection caused by the gravitational force acting on the bead[33].

During the imaging of biological samples in air the sample may be irreversibly damaged owing to strong adhesional forces between the probe and sample. These forces are due to a thin layer of water, which covers the surface of most samples. At small probe sample separations a neck of water is formed between the probe and sample, thus generating large probe–sample adhesive forces. Weisenhorn *et al.*[34] noted that these 'capillary' forces could be reduced by immersing the sample in liquid. Similarly, most force measurements are performed in liquid so that the large capillary forces do not mask the forces under study.

The study of biomolecular interactions using the AFM

Biological and biochemical processes are governed by a multitude of molecular interactions. Examples of processes mediated by molecular recognition are enzyme–substrate interactions, antigen–antibody interactions and many drug–target associations. This dependence on specific interactions is also true for an increasing number of modern biosensors and chemically or biologically engineered biomaterials. Specific

molecular interactions, such as those between receptor molecules and their specific ligand molecules, have bond energies that lie between those typical of an ionic bond (e.g. sodium chloride) and van der Waals interactions (e.g. between atoms of a noble gas)[20]. Lee *et al.*[20] estimated that a force of approximately 10^{-9} N was required to break a receptor–ligand complex.

To achieve force measurements between individual receptor–ligand complexes, the AFM probe is typically functionalized with a biomolecule of interest and its complementary molecule immobilized onto the sample surface (Fig. 3). Force measurements are then recorded between the functionalized surfaces. During the approach trace of the force measurement the receptor and ligand molecules are brought into contact, allowing receptor–ligand complexes to form. The 'sticking force' (Fig. 2) observed as the probe and surface are separated corresponds to the force required to separate the receptor–ligand complexes. Because the AFM probes used in such experiments typically have an apex radius of 10–50 nm (Ref. 14), the number of receptor–ligand complexes formed during a force cycle can be as low as one or two molecular pairs.

To determine exactly the number of interacting molecules during each force measurement a prior knowledge of the surface density of the immobilized molecules, probe geometry and probe–sample contact area is required. Protein surface coverage can be assessed relatively easily using methods such as radiolabelling[20] and fluorescence techniques[29]. However, an exact determination of probe geometry and probe–sample contact area is more problematical. In an attempt to overcome such problems, various groups[20,22,28] have attached smooth micrometre-sized spheres (radius of 2–10 μm) to the ends of cantilevers and employed them as probes with controlled geometry; this allows a greater control of probe–sample contact area.

In experiments employing spherical probes, the probe–sample contact areas are larger than those in similar experiments employing functionalized AFM probes. Consequently, many receptor–ligand complexes can be formed on contact, and hence the adhesive forces in such experiments may correspond to the rupture of a number of receptor–ligand bonds. The rupture force for a single receptor–ligand interaction may be obtained by blocking out the majority of interaction sites on one of the surfaces[21], by the addition of known or unknown quantities of receptor or ligand to the experimental system.

The streptavidin–biotin[35] system was the initial receptor–ligand interaction to be studied using this technique. Streptavidin, a 66–75 kDa protein, comprises four identical subunits each containing a single biotin binding site[36]. The high specificity and affinity (binding constant, $K_d = 10^{-15}$ mol l^{-1}) of the complex, and the availability of structural[37] and thermodynamic[38] data made it an ideal model system for the study of receptor–ligand interactions. Lee *et al.*[20] used biotin-functionalized glass beads attached to AFM cantilevers and streptavidin-coated mica surfaces, and estimated the strength of a single streptavidin–biotin

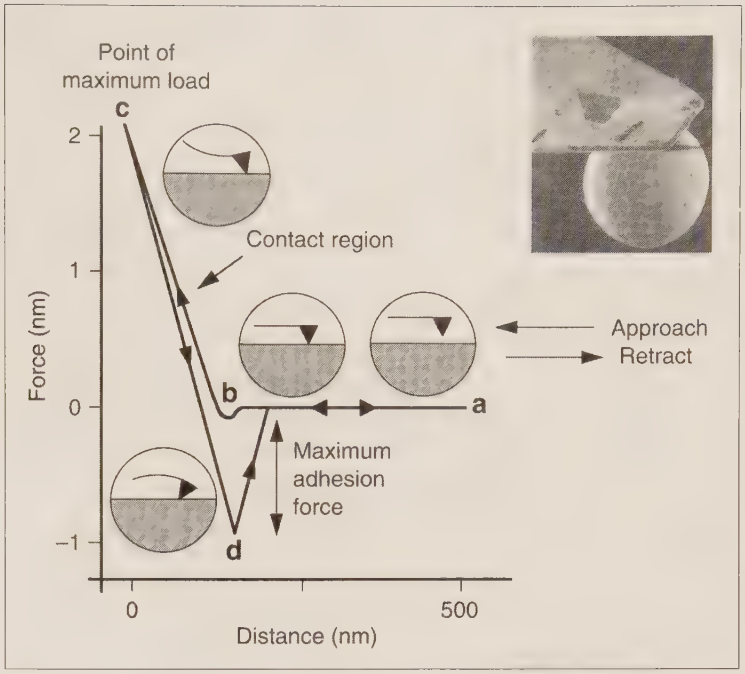

Figure 2

At the start of a force measurement (**a**) the probe–sample separation is large and the cantilever is at an equilibrium or rest position (zero cantilever deflection). The probe–sample separation is reduced (approach trace) at a constant velocity. At a point close to the sample surface, the probe jumps into contact with the sample surface (**b**). This 'jump to contact' is observed when the attractive force gradient exceeds the stiffness or spring constant of the cantilever spring. Forward motion continues until a predetermined point of maximum load is reached (**c**). The direction of motion is then reversed so that the probe and sample are separated. A plot of cantilever deflection against distance moved by the *z*-piezo is obtained as raw data. As the probe is separated from the sample surface (retract trace) the probe 'sticks' to the surface due to interactions between the probe and the sample (**d**). The magnitude of this 'sticking' force is calculated from the difference between the maximum cantilever deflection (d) during the withdrawal phase of the cycle and the point of zero cantilever deflection (a). Inset: a scanning electron microscope image of a tungsten bead (10 μm diameter) attached to the apex of an atomic force microscope cantilever.

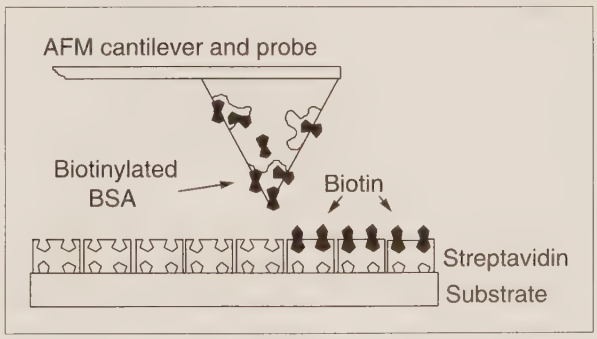

Figure 3

A schematic diagram of an atomic force microscope probe functionalized with biotinylated bovine serum albumin (BSA) and a streptavidin-coated substrate. To achieve force measurements between individual receptor–ligand complexes, the AFM probe is functionalized with a biomolecule of interest, e.g. biotin, and its complementary molecule immobilized onto the sample surface, e.g. streptavidin. Force measurements are then recorded as the probe and sample are brought in and out of contact.

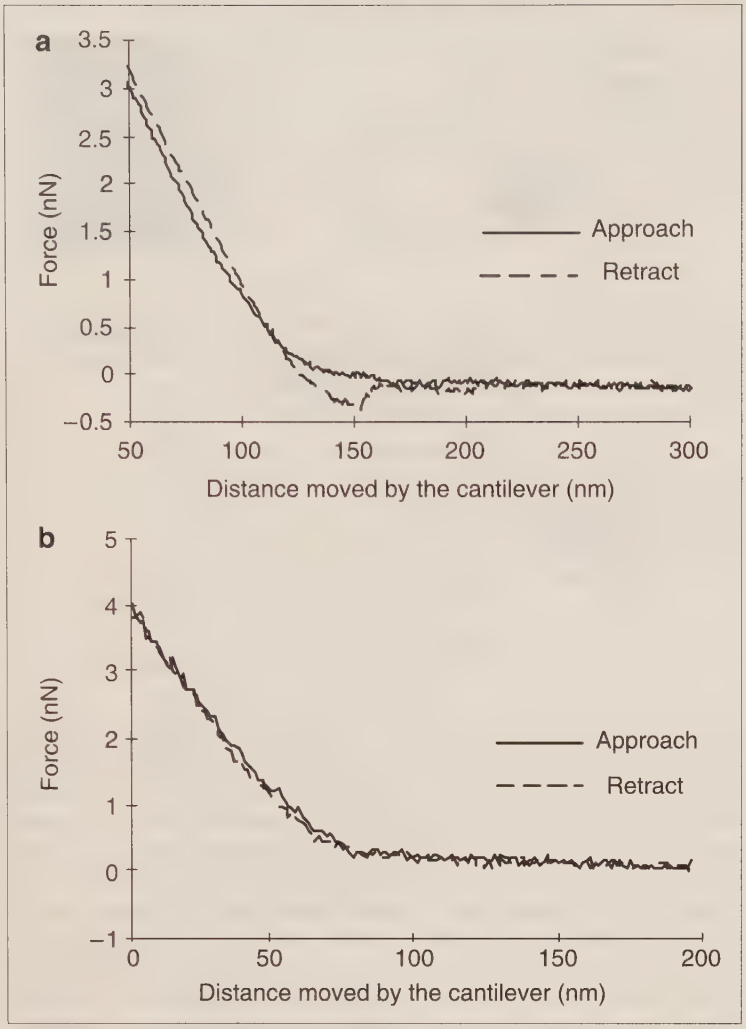

Figure 4

(**a**) A force measurement between a biotinylated AFM probe and a streptavidin-coated immunoassay well. A sharp 'sticking force' of approximately 300 pN, typical of a specific tip–sample interaction, is observed in the retract trace. (**b**) After flooding the experimental system with excess ligand, adhesion points are not observed in such force curves.

bond to be 340 ± 120 pN. A similar approach was adopted by Florin *et al.*[21], who measured quantized forces of interaction between a biotinylated agarose bead and an AFM probe functionalized with avidin, a 67 kDa protein closely related to streptavidin.

Using this technique, it was also shown that the AFM can discriminate between different analogues of biotin[25] and streptavidin[26]. In such experiments the force data was related to thermodynamic data for the formation of the receptor–ligand complexes. To date, the rupture force appears to be correlated with the enthalpy, rather than the free energy of binding, although this observation is very much still under discussion[25,26].

Recently, Allen *et al.*[31] have measured adhesive forces between biotinylated AFM probes and commercial immunoassay surfaces coated with streptavidin, illustrating that the AFM can also be used to monitor biomolecular interactions on real biosensor surfaces. Figure 4a displays a typical force curve obtained between a biotinylated AFM probe and a streptavidin-functionalized immunoassay surface. When using the AFM to measure the forces required to separate receptor–ligand interactions it is essential to perform control experiments to ensure that the measurements correspond to the specific interaction under study and not to any other factors[30]. For example, to confirm that streptavidin–biotin binding was causing the 'sticking force' observed in Fig. 4a, the experimental system was flooded with excess ligand so that the biotin binding sites on the well surface were blocked. Force measurements recorded thereafter displayed no points of adhesion, showing that the biotinylated probe could no longer bind with the streptavidin-coated surface (see Fig. 4b).

Similar experimental approaches have been employed to study the interactions between complementary strands of DNA (Ref. 22), cell adhesion proteoglycans[27] and antibodies with their specific antigens[28–30]. Thus the adaptability of this technique in the study of biomolecular interactions is illustrated.

Probing the nanomechanical properties of biological samples

In addition to measuring adhesion forces between a probe and sample, it is also possible to use the AFM probe and cantilever system to investigate the 'stiffness' or elastic properties of a sample. Such information may be obtained from the gradient of the approach trace in the contact region of the force curve (Fig. 2). Initial studies were performed by Tao *et al.*[39], who studied the microelastic properties of hydrated cow tibia. They observed that the gradient of the line in the contact region of the force curve was greater for hard materials, such as steel (high modulus of ridigity), than for soft materials, such as rubber (low ridigity modulus). By imaging a section of hydrated cow tibia at different imaging forces, and performing force measurements at different points on the sample surface, they observed dramatic variations in the elastic properties of a sample surface across distances as small as 50 nm.

Radmacher *et al.*[40] used a similar experimental approach to image a soft gelatin film on mica. The resolution of AFM images obtained in pure water was poor, owing to the deformation of the soft sample by the AFM probe. By adding increasing amounts of propanol to the imaging media the resolution of the images was found to improve, with the best resolution obtained in pure propanol. Force measurements revealed that the elastic modulus of the film was higher in propanol than in water. Therefore, the improvement in image resolution was attributed to the increase in elastic modulus of the sample as the amount of propanol in the imaging media increased.

Hoh and Schoenenberger[41] have imaged monolayers of viable Madin–Darby canine kidney (MDCK) cells. During glutaraldehyde fixation of the monolayer, AFM measurements revealed an increase in the stiffness of the cells with time. The potential of such measurements to provide information that may relate the cytoskeletal architecture of cells to biological function was illustrated by Radmacher *et al.*[42], who

studied the elastic modulus of human platelets, *in vivo*, using the AFM. Depending on the position of the AFM probe on the cell surface the elastic modulus was found to lie in the range 1–50 kPa.

Two-dimensional mapping of surface forces

The measurement of surface forces at single points on a sample surface prompted the development of various methods that would produce two-dimensional maps of interaction sites on a sample surface. A technique known as chemical force microscopy was developed by Frisbie *et al.*[43] in which a gold-coated probe was functionalized with a hydrophilic chemical species. When scanned over a surface patterned with discrete areas of hydrophobic and hydrophilic chemical species, images were produced that were dependent on the chemical nature of the probe–sample interaction.

Instruments that perform force measurements at each imaging point on a sample surface allow the production of two-dimensional maps of surface forces acting at different probe–sample separations. For example, Radmacher *et al.*[44] have produced maps of adhesion and elasticity of lysozyme molecules on an uncovered substrate. In that case the image contrast arose because the probe–sample adhesional forces were much larger on the uncovered substrate than on the lysozyme molecule, and the protein molecules were 'softer' than the underlying substrate.

Of recent interest is the use of biomolecule-functionalized AFM probes for the mapping of specific recognition sites on a sample surface. In studies by Hinterdorfer *et al.*[29] antibody molecules were attached to AFM probes using a long molecular tether. Using such probes it was possible to determine the spatial distribution of surface immobilized antibody binding sites.

Future prospects

The further development of this technique will not only impact on our fundamental understanding of molecular processes but may also aid in the identification of high-affinity lead compounds in the process of drug discovery at its first and most difficult stage. Interestingly, the development of a biosensor based on the force microscope technology outlined in this review has recently been reported[45]. Owing to the high sensitivity of AFM cantilevers the biosensor can detect biological species at concentrations of 10^{-18} mol l^{-1}, which is approximately eight orders of magnitude more sensitive than conventional techniques.

Acknowledgements

The authors would like to thank Johnson and Johnson Clinical Diagnostics, Chalfont St Giles, Buckinghamshire and the EPSRC for the funding of S. A., and in particular J. Davies, A. C. Dawkes and J. C. Edwards for their assistance and guidance during the experimental contributions to this manuscript. The scanning electron microscopy was performed at SmithKline Beecham Pharmaceuticals, Surrey, by J. Warrack and A. P. Shivji. P. M. W. is funded by an EPSRC SPM Initiative Grant.

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Vaccine strategies: selective elicitation of cellular or humoral immunity?

Gene M. Shearer and Mario Clerici

The discovery of immunoregulatory cytokines and the fact that they modulate the cellular and humoral arms of the immune system, generally in opposing directions raises fundamental questions concerning vaccine development. Because antibiotic-resistant infectious organisms are appearing at an increasingly rapid rate, more emphasis will need to be placed on prevention of infection/disease via immunization and less on post-infection antibiotics. To accomplish this task effectively, immune regulation should be integrated into vaccine design. Here we consider the opposing potentials of cytokine-regulated cellular and humoral immunity, and question whether the 'best of both worlds' is possible or desirable.

The immune system can be divided into two broad functional categories: humoral immunity (HI) and cellular immunity (CI) (Ref. 1). HI is effected by antibodies, bivalent protein molecules that can be cell-bound or cell-free. Antibodies bind to antigenic determinants, usually on the surface of viruses, bacteria or parasites, and inactivate these infectious agents. Antibodies are primarily effective against extracellular infectious agents. By contrast, CI is mediated by effector cells that destroy infected cells of the host by direct cell-to-cell contact, or by the release of molecules that possess killing activity. In this way CI mechanisms focus on destruction of the source of infection, and are more effective against intracellular infections. CI is less clearly understood than HI and appears to be more complex.

Both CI and HI require a special type of T cell to initiate and/or enhance cellular and humoral responses. These cells are called T helper (Th) and produce protein molecules known as cytokines that are responsible for the helper effects. Thus, both the HI and CI components of the immune system are dependent on Th, which actually comprises part of the cellular arm of immunity. Using cell cloning techniques, Mosmann and Coffman originally identified two functionally-distinct Th subsets in the mouse[2]. These cloned helper cells, called Th1 and Th2 cells produced, respectively, interferon-γ (IFN-γ) and interleukin-4 (IL-4), and provided helper activity primarily for CI and HI. Similar findings were subsequently made in the human[3]. These above investigators and their colleagues also found that Th1 and Th2 responses were regulated not only in a positive but also a negative way such that cellular responses were augmented by IL-2 and IFN-γ but decreased by IL-4 and IL-10. In contrast, antibody responses were enhanced by IL-4 and IL-10, but were down-regulated by IFN-γ[4] and another cytokine, IL-12, that enhances IFN-γ and is produced by monocytes[5]. The above summary, however, is oversimplified in that not all antibodies are enhanced by Th2 clones. For example, the IgG2a subclass can be enhanced by Th1 clones.

Because these regulatory cytokines can be produced by different cell types and the *in situ* human immune system is more complex than a clone of T cells, we have interpreted and expanded the Th1/Th2 hypothesis to the type 1/type 2 model that includes all cell types that produce these cytokines[1]. Thus we characterize the immune response of an individual on the basis of whether there is a dominant cellular or humoral response with the associated production of IL-2, IL-12 and/or IFN-γ or of IL-4, IL-5, IL-6 and/or IL-10, respectively (see Fig. 1). Box 1 also compares the Th1/Th2 model that is based on cloned $CD4^+$ T cells with the type 1/type 2 model derived from the concept that multiple cytokines produced by different cell types contribute to the overall balance or dominance of cellular or humoral immunity.

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PII: S0167-7799(97)01011-1

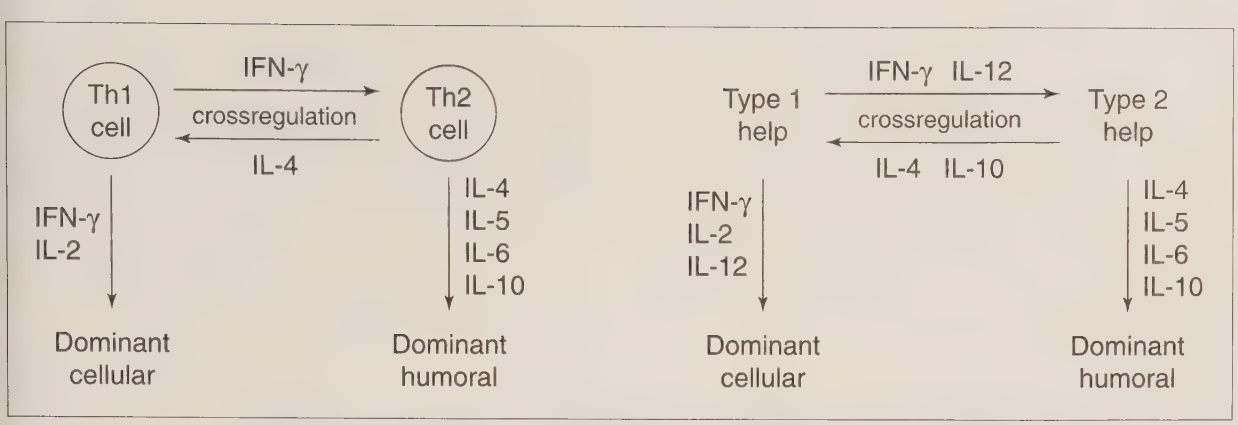

Figure 1
Comparison of the Th1/Th2 model of cloned CD4+ T cells with the type 1/type 2 model for generation of dominant cellular or humoral responses.

Kinetics of cellular and humoral responses

The primary immune response against antigens of infectious agents characteristically involves a cellular effector response followed by a humoral response (Fig. 2a). A typical secondary response resulting from previous immunization (either natural or induced) is shown in Fig. 2b. The secondary response develops more rapidly and is more potent than the primary response. The decline in CI that occurs concomitantly with the increase in HI may be owing to the down-regulatory potential of type 2 cytokines, such as IL-4 and IL-10, that not only increases HI but also can reduce CI. This decline in CI during progression of the immune response may have two important effects. First, it may serve to keep the CI in check, thereby reducing the possibility of CI and type 1 cytokine-induced autoimmune conditions, for example Crohn's disease and type 1 diabetes. However, if CI is the major protective arm of the immune system, such as in chronic intracellular infections, the shift from a dominant cellular to humoral response could compromise protective CI, resulting in recurrence of an infection, to which the cellular arm of the immune system had initially gained control[6]. Primary infection with HIV may be such an example, because the kinetics of cellular and humoral responses are similar to those of Fig. 2a and the serum viral titer drops by a factor of 10^2–10^4 just after the acute infection phase, at the time of maximum cellular effector activity and prior to the appearance of neutralizing antibody and the decline in CI (Ref. 7).

Figure 2c also shows a situation in which immunization can result in CI without, or with minimal, HI. Selective immunization of the cellular arm has been seen in both animals[8,9] and humans, including immunization with HIV antigens[10,11]. This example of selective immunization of CI is characterized by low dose antigen exposure or infection, and could provide an approach for preferential immunization of CI (Refs 12, 13). We are not aware of dose-related immunization conditions that elicit HI without also inducing CI, although the kinetic patterns shown in Fig. 2a and 2b suggest that antibodies can dominate later in the immune response.

Conditions for cellular versus humoral immunity

Until very recently, vaccine strategies have not considered the design of vaccines that would preferentially elicit either CI or HI. Therefore, published information on this topic is limited. Box 2 lists several parameters that may contribute to a dominant cellular or humoral response, although the conditions under which some of these parameters result in a preferential response of one type or the other have not yet been determined.

As mentioned above, low dose immunization can result in a dominant cellular response without appreciable antibody production. This phenomenon has been observed in non-viral animal models[8] and in macaques exposed to SIV (Ref. 9). Immunization with higher doses of antigen results in both CI and HI, with HI often persisting after CI has waned (see Fig. 2). Notably, low dose inoculation of mice with protozoan parasites followed by high dose challenge has been reported to result in only a cellular response,

Box 1. Comparison of Th1/Th2 and type 1/type 2 models

Model	Cellular source of cytokines	Description of model
• Th1/Th2	• CD4+ T	• Tests for cytokines produced by cloned CD4+ T cells
• Type 1/ type 2	• CD4+ T; CD8+ T; monocytes; B cells	• Tests cytokine effects produced by multiple cell types on immune function

Box 2. Factors in vaccine design that can influence dominant cellular versus humoral responses

- Dose of antigen
- Adjuvant used
- Route of immunization
- Type of antigen
- Type of antigen presenting cells
- Costimulatory signals
- Genetic background of vaccinee
- Cytokine environment
- Immunologic status of vaccinee
- Age of vaccinee

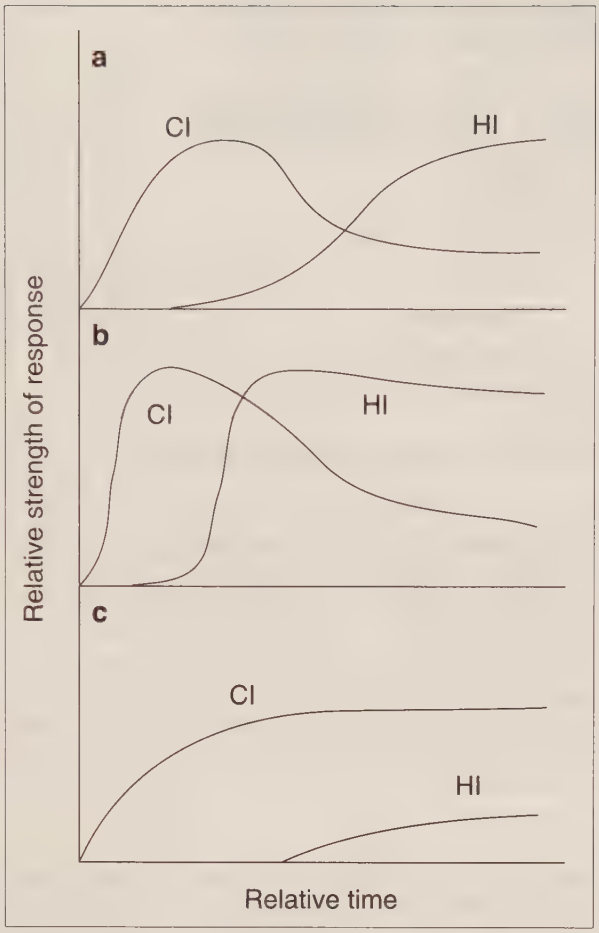

Figure 2
Kinetics of cellular immunity (CI) and humoral immunity (HI). (**a**) Characteristic primary immune response illustrating dominant CI followed by dominant HI. (**b**) Typical secondary immune response showing similar dominant CI followed by dominant HI, but with more rapid kinetics and stronger responses than in primary response. (**c**) Atypical strong and persistent CI in the absence of or with weak HI, resulting from low-dose antigen immunization.

despite the fact that a primary high dose response elicits a dominant HI (Refs 14, 15). This phenomenon has been termed 'low dose lock-in', and suggests that, once initiated, a selective cellular response is not easily switched to a strong humoral response. Thus, a mouse strain that is genetically susceptible to leishmaniasis and to tuberculosis can be protected by low dose immunization and CI against subsequent high infectious doses that would normally result in antibody production and disease[14,15]. It is possible that HIV-exposed individuals who remain seronegative but exhibit cellular responses were initially exposed to a low dose of HIV antigens, and that HIV-specific CI was dominant, despite subsequent exposures to high viral doses that might otherwise elicit a strong humoral response[13].

Adjuvants can contribute to directing a response towards dominant CI or HI. Alum, the major adjuvant approved for human immunization, is known to elicit strong antibody responses. Adjuvants based on bacterial products, such as mycobacteria, are considered to be strong potentiators of cellular responses. Other adjuvants are currently under study for their ability to elicit dominant CI or HI or both types of immunity. Antigens presented in liposomes have been found to elicit strong CI, and monophosphoryl lipid A has been reported to be an adjuvant that generates strong and persistent cellular and humoral immunity[16]. Cytokines can also be used as adjuvants administered either separate from the vaccine or inserted as part of the genetic element of the immunizing material.

The type of antigen-presenting cells (APCs), preparation of the vaccine, route of immunization and co-stimulatory signals can all influence dominant cellular or humoral responses. It is generally considered that APCs of the monocyte/macrophage lineage present antigen that favors dominant CI, whereas B cells present antigen that elicits dominant HI (Ref. 17). Genetic background of the vaccinee may also influence CI and HI. For example, the BALB/c and C57BL/6 inbred mouse strains exhibit dominant humoral and cellular responses after infection with several different parasites and bacteria[15]. However, it is doubtful that such distinct genetic differences will be observed in outbred humans. Age may also influence immune potential, as well as CI and HI. It appears that the potential for dominant humoral immunity may exist in infants and possibly in aged animals and humans[18].

Several different types of vaccine preparations have been used for immunization, including whole killed organisms, synthetic peptides of antigens from infectious agents, attenuated viruses, antigens packaged in recombinant viral vectors and naked DNA[19]. Any of the above may be able, under appropriate conditions, to elicit dominant CI, HI or both, depending on the adjuvant, antigen dose, route of immunization and cytokine profile of the vaccinee.

Consequences of simultaneous strong CI and HI

Although optimal vaccine strategy may dictate that we strive for maximum simultaneous CI and HI, the tendency of the immune system to self-regulate by reducing one arm while increasing the other may be providing a subtle but important message. Would maximizing both the cellular and humoral components of immunity initiate a runaway immune system that would result in autoimmune diseases? There is little doubt that we can and eventually will achieve the 'most of both worlds', through genetically engineered vaccines and adjuvants, cytokine-redirected immune responses and DNA immunization. We should, however, be aware that the 'most of both worlds' does not necessarily translate into the 'best of both worlds', as it is likely that the natural course of an immune response has had a selective evolutionary advantage for the individual.

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Integrated approaches to the design of media and feeding strategies for fed-batch cultures of animal cells

Liangzhi Xie and Daniel I. C. Wang

Animal cell culture has become an important approach for the production of biologically functional proteins for human therapy. The quantity and quality of protein production are influenced by the culture environment, which is subject to change over the course of cell cultivation. Therefore, it is vital to design an optimal culture environment and control it within an optimal region to maximize the productivity. This requires that the factors affecting the culture environment (nutrient concentrations, by-product accumulation, pH and osmolality) and cell growth be integrated into the design of culture media for fed-batch animal cell cultures.

The capability to secrete properly folded proteins with post-translational modifications, such as glycosylation, makes animal cells the ideal hosts for the production of various proteins with biological functions for diagnosis and therapy. In industry, animal cell cultivation has been practised most often in a batch mode. However, many other operating modes, such as fed-batch, continuous and perfusion, have been extensively studied. Simplicity in process control and operation, resulting in process consistency, low contamination rate and low operating costs are some of the advantages of batch culture; the disadvantages are low product titer and productivity, which are two important parameters that one wants to maximize. The desired product is secreted by viable cells, hence product concentration is directly proportional to the integrated viable cell concentration with time. Obviously, a high viable cell density maintained for a long time is required to maximize the product concentration. However, this mainly depends upon the composition of the medium employed. It is well known that animal cells require many essential nutrients to survive *in vitro*. These include glucose, essential amino acids, vitamins, serum components and inorganic salts. Cell growth not only consumes nutrients but also generates by-products such as ammonia and lactate, which are known to be toxic to animal cells, and NAA (see Glossary). The accumulation of NAAs may alter the medium osmolality. In a batch culture, the theoretical maximum cell density is limited by the amount of nutrients present in the initial medium. Therefore, the nutrient level in the culture medium needs to be increased. However, the actual maximum cell density depends on many other parameters. The depletion of key nutrients,

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PII: S0167-7799(97)01014-7

Glossary

CHO – Chinese hamster ovary cells
K_m – Monod constant, mM
NAA – non-essential amino acids
X_i – molar fraction of the *i*th nutrient in the feed medium
$\theta_{a,i}$ – stoichiometric coefficient for the *i*th amino acid (i = 1,20), mmole/cell
θ_{ATP} – stoichiometric coefficient for ATP demand, mmole/cell
θ_{glc} – stoichiometric coefficient for glucose, mmole/cell
θ_i – stoichiometric coefficient for the *i*th nutrient in the feed medium, mmole/cell
θ_k – stoichiometric coefficient for the *k*th by-product, mmole/cell
θ_p – stoichiometric coefficient for product formation, mg/cell
$\theta_{v,j}$ – stoichiometric coefficient for the *j*th vitamin (j = 1,10), mmole/cell
ΔN_t – net increase of total cell number, cells
$\Sigma\theta_i$ – total stoichiometric coefficient of all nutrients in the feed medium, mmole/cell

accumulation of toxic by-products or changes of pH and osmolality can lead to cell death. The design of a proper culture medium is thus critical to achieve optimal cell growth. Furthermore, the protein quality (macroheterogeneity and microheterogeneity in glycosylation) is influenced by the culture environment[1,2].

Motivation for medium design

Since Eagle[3] developed the minimum essential medium (MEM) for animal cell growth, empirical trial and error methods have been practised in the subsequent development of culture media. The fact that more than 30 essential nutrients are required for cell survival renders the optimization of medium composition difficult; the depletion of just one essential nutrient could result in the cessation of cell growth followed by cell death[4]. At the end of a batch culture, significant amounts of most of the essential nutrients are often still present in the medium, whereas some may be completely exhausted. This indicates that the composition of the basal medium is not optimal to achieve a high cell density, even though cells may grow perfectly well at low densities.

A simple attempt to circumvent nutrient depletion is to elevate the nutrient concentrations in the medium, without altering the medium osmolality, by modifying the salt concentrations accordingly. Considerable improvement in cell density and product titer was attained in a hybridoma culture using a fortified RPMI 1640 medium[5]. This is not a generic approach as Chang *et al.* have shown that high glucose and glutamine concentrations lead to high lactate and ammonia production[6]. The dilemma between nutrient depletion and by-product formation cannot be solved in a batch culture system.

Fed-batch cultures have been successfully carried out to extend the culture span in many cases. Clearly, fed-batch culture possesses many advantages over batch culture: nutrient feeding prevents its depletion, and dramatic changes of the nutritional environment can be significantly lessened through balanced feeding. In addition, it is no longer necessary to include high nutrient concentrations in the initial medium, since the demand can now be satisfied by a supplement medium through feeding. One can further separate the nutritional needs for maintaining a reasonable cell growth rate from the stoichiometric needs for cell replication. This makes it possible to solve the dilemma to achieve a high cell density.

Design of culture environment

Most commercial media are designed for the purpose of achieving a maximum cell growth rate. The effects of cell growth on the culture environment are often neglected. This usually results in a dramatically changed culture environment that leads to early cell death even though sufficient nutrients are still available in the medium. Product concentration increases exponentially with time because of the exponential cell growth. Obviously, viable cell density and culture span are more important than the growth rate if the productivity needs to be maximized.

The factors affecting lactate and ammonia formation need to be integrated into the design of the culture environment. This issue becomes more critical at a high cell density where the amount of by-products generated in even a short period can be problematic. This is because the accumulation of by-products (ammonia and lactate) also increases exponentially with time if a constant specific product rate is assumed for the by-product formation. The production of both lactate and ammonia was found to be significantly reduced at low glucose and glutamine concentrations in cultures with human diploid foreskin cells (FS-4) (Ref. 7) and hybridomas[8–10]. The ratio of lactate-to-glucose increases with glucose concentration in the medium[11] and cell growth rate[12]. Recently, a study with $^1H/^{15}N$ NMR in HeLa and CHO cell cultures showed that ammonia was produced predominantly from the glutamine amide group[13]. These results suggest that glucose and glutamine be maintained at very low concentrations to avoid massive lactate and ammonia production. Cell growth kinetic studies reveal that the Monod constant (K_m) is about 0.5 mM for glucose[14] and 0.2 mM for glutamine[15]. In fed-batch cultures, the concentrations of glucose and glutamine in initial media should be around twice their K_m values to ensure low lactate and ammonia production rates while maintaining a cell growth rate at 70–90% of its maximum.

Recently, it was found that glutamine-based dipeptides (alanyl-glutamine and glycyl-glutamine) could replace free glutamine in culture media[16,17]. These dipeptides can be hydrolyzed by a cytosolic peptidase, releasing free glutamine for cell growth. The advantage of this approach is that the dipeptides are resistant to non-enzymatic deamination even at high temperature, and hence are less ammoniagenic. This allows long-term storage and autoclaving as a means for medium sterilization. However, free glutamine is

rapidly released into culture medium, as shown by the increase in glutamine concentration shortly after the inoculum[17]. Non-enzymatic glutamine deamination still occurs during the culture process. Hence, the reduction in ammonia production from glutamine is limited. This approach also has the disadvantage that a significant amount of alanine or glycine is formed from these dipeptides while the requirement for cell growth is much smaller as compared with that of glutamine. Accumulation of alanine or glycine is inevitable. In high cell density cultures where the total glutamine consumption can be as high as 75 mM (Ref. 10), the amount of alanine or glycine generated from these dipeptides will elevate the osmolality to a detrimental level. Other approaches to reduce ammonia production include complete elimination of glutamine from culture media via adaptation procedures[18] or transfection of a glutamine synthetase system[19]. Almost no ammonia is produced with glutamine-free medium[20]. However, it should be noted that not every cell line can grow in glutamine-free media[21].

Balanced nutrient composition in feed medium for fed-batch cultures

Conventional approaches

The performance of a fed-batch culture relies not only upon the initial medium but also on the control of the dynamic culture environment resulting from the consumption of nutrients and accumulation of by-products. The design of a balanced nutrient composition in the feed medium is thus critical, which allows one to control the entire nutritional environment through the feeding flow rate.

Concentrated amino acids and vitamins were employed by Luan *et al.*[22] in an attempt to elongate culture span. The effects of the addition of various combinations of glutamine, essential amino acids, vitamins and serum were studied. Owing to the omission of glucose in the feed medium and the empirical protocol, improvement in the culture performance was limited. Nevertheless, this early attempt stimulated numerous studies on the design of supplemental media for fed-batch cultures. Realizing the central roles of glucose and glutamine in energy metabolism and by-product formation, Glacken and co-workers[15] used an empirical approach to determine the glucose and glutamine concentrations in a 4 × concentrated complete medium (DMEM without salts except sodium chloride) for feeding, to prevent glucose or glutamine depletion. A mathematical model was developed to determine a feeding policy.

Using 10 × concentrated IMDM without salts for feeding in a culture of NS0 cells transfected with a glutamine synthetase vector system, Bibila and co-workers[23] achieved a sevenfold increase in product titer. The unique glutamine synthetase vector eliminated the ammonia production and the requirement for glutamine. Because the nutrient ratios in the basal medium were not balanced with the demands for cell growth, accumulation of some nutrients and by-products was observed, which resulted in a significant increase in osmolality. This approach was improved through iterative analysis of the spent culture media to optimize the nutrient ratios in the feed medium[24]. Protein-free lipid emulsions were also included in the supplement medium. Although the design of the concentrated feed medium and feeding policy were empirical, substantial improvement was achieved in final antibody titer.

In an attempt to balance the nutritional demands for cell growth, nutrient consumption rates were measured in batch cultures to determine the ratios of key nutrients (glucose and some amino acids) in the feed medium[25]. This provides an effective, rapid and simple approach to the nutrient depletion problem. The disadvantages of this approach are obvious: nutrient consumption rate is affected by culture environment, and hence may not be applicable to different conditions. More importantly, batch culture is not an efficient process, owing to the enormous production of by-products[26].

An integrated approach for medium design

The stoichiometry governing microbial fermentation is often simple and has been the subject of detailed studies[27]. However, the stoichiometric nutritional demands for animal cell growth have only recently been quantified. The understanding of the cellular needs for nutrients will allow the quantitative design of culture media in such a way that depletion or accumulation of nutrients can be avoided or reduced. However, the determination of the stoichiometric equation governing animal cell growth is not an easy task, mainly owing to the sophisticated nutritional demands and the metabolic network.

In microbial systems, metabolic flux analysis has been applied to examine flux distributions[28–31] and to optimize product formation[32,33]: intracellular flux analysis in hybridoma cells was performed by Zupke and Stephanopoulos[34]; the primary metabolic pathways in hybridomas were studied by Mancuso *et al.*[35] and by Sharfstein *et al.*[36] in a hollow fiber bioreactor; and a hybridoma metabolic reaction network was employed by Savinell and Palsson[37] to identify the limiting nutrient in a cell culture process.

The progress made in the understanding of the intracellular metabolic reaction network makes it possible to define the stoichiometric equation governing animal cell growth (Eqn 1, in Fig. 1). In an ideal system, where there is no by-product (lactate, ammonia and non-essential amino acids) formation, a detailed study on the intracellular metabolic reaction network leads to the correlation between products (cell mass, product and energy requirement) and nutrients (glucose and amino acids). Hence, the stoichiometric coefficients for the nutrients are completely defined by the cell composition, product formation and energy requirement[38]. Unfortunately, in a real system, the variable by-product formation caused by variations in culture environment or operating conditions renders the metabolic system undefined. Hence, there is no unique solution to the metabolic reaction network.

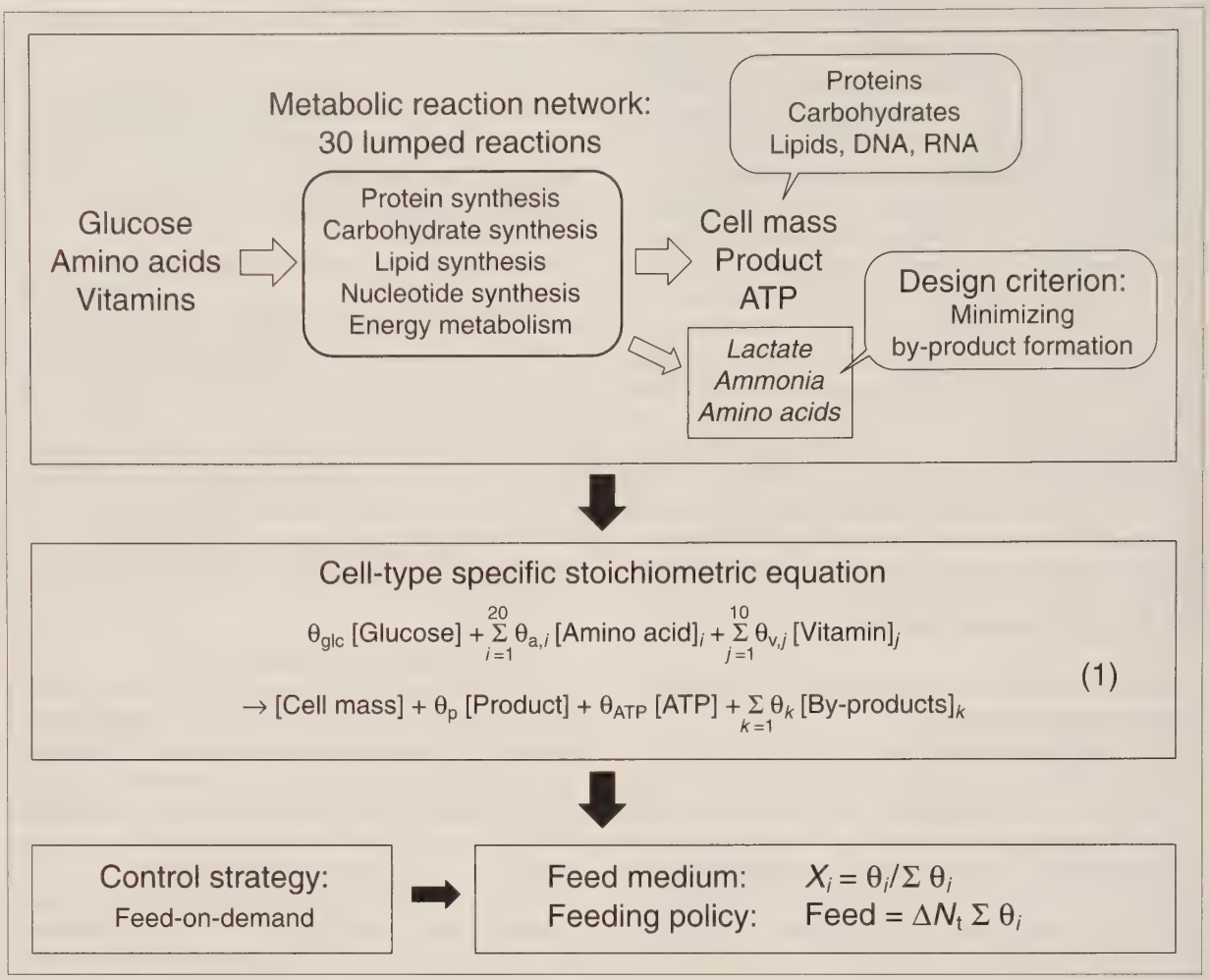

Figure 1
Outline of an integrated approach to medium design and nutritional control in fed-batch cultures.

The implementation of a design criterion that the formation of by-products (lactate, ammonia and non-essential amino acids) should be minimized allows one to determine the minimum nutritional needs for an optimal cell growth[9]. Supplemental media were formulated for feeding in fed-batch cultures according to the stoichiometric coefficients of the nutrients. A feeding strategy was also derived from the stoichiometric analysis to allow the control of the nutritional environment (glucose, essential amino acids, some non-essential amino acids and vitamins). This integrated stoichiometric approach is summarized in Fig. 1.

Application of the stoichiometric approach in hybridoma cell cultures led to significant increases in cell density, culture span and product concentration as well as a dramatic decrease in by-product formation[9,10,39]. In a bioreactor fed-batch hybridoma culture, the key nutrient concentrations were maintained at low levels with automatic stoichiometric feeding of a balanced supplement medium[10]. The maximum viable cell density reached 1.7×10^7 cells/ml; culture life was extended to 550 h; specific lactate production rate was reduced by 80-fold (less than 5% glucose was converted into lactate as compared with 80% in a batch); specific ammonia production rate was reduced by sixfold; antibody concentration was increased by 50-fold as compared with a batch culture. Material balance studies indicate that this integrated stoichiometric approach leads to more efficient nutrient utilization and energy production[26]. With modifications, this approach was also successfully applied in a microbial system[40].

Future prospects

Conventional continuous and perfusion cultures have the advantage of high productivity but suffer from low product concentrations caused by the high dilution rate required to maintain cells viable. The problem can be solved by designing a stoichiometrically balanced medium with high concentrations of essential nutrients to reduce the dilution rate. This will substantially increase the viable cell density, product concentration and productivity.

Serum-free medium is desirable for animal cell growth, owing to the problems associated with serum supplementation. Supplements such as growth factors, binding proteins, trace metals, protecting agents and fatty acids are often found in serum-free media[41]. However, the quantification of the requirements for

these supplements has not been studied yet. The understanding of the quantitative demands for growth hormones, and other essential components in serum-free media, will allow high productivity to be attained under a chemically defined condition.

Conclusions

Medium design is equally important as the operation of an animal cell culture process. Quantification of the minimum nutritional needs for cell growth allows the supplementation and consumption to be balanced. Consequently, the culture process can be controlled to avoid nutrient depletion or accumulation. This improves the culture performance and the reproducibility of the culture process. The understanding of the quantitative demands for growth factors, trace elements, etc. will further advance the technology for the production of therapeutic proteins using animal cells under completely defined conditions.

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Techniques for long life

Culture of Immortalised Cells

edited by R. Ian Freshney and Mary G. Freshney, John Wiley & Sons, 1996. UK£40.00 (389 pages) ISBN 0 471 1213 47

Cells that are cultured from normal somatic tissue do not live for ever: individual cells undergo a set number of divisions (the Hayflick limit) before they senesce and die. By contrast, cells that are cultured from malignant tumours do not have a limited lifespan and, given the right culture conditions, will grow indefinitely. Sometimes normal cells will undergo a malignant transformation *in vitro* and, when this happens, they lose the phenotypic characteristics of normal cells and develop a malignant phenotype with unlimited growth. At one time it was assumed that transformation to a malignant phenotype was essential for the development of immortalized cell lines. Recently, however, techniques have been developed that allow normal cells to become immortalized in culture whilst retaining at least some of the 'normal' phenotypic characteristics. Such cells are invaluable in biotechnology, not least because they can carry out the post-translational modifications to genetically engineered proteins which are unavailable in prokaryotic systems. Immortalization of normal cells is achieved by infecting cells with viruses, such as simian virus 40 (SV40) or Epstein–Barr virus (EBV) (the latter for immortalizing B cells), or by transfecting the cells with immortalizing genes, such as the SV40 large T antigen gene.

Culture of Immortalised Cells is the third book in a series that deals with the culture of specialized cells. It is a truly international book with contributions from specialists around the world. The book begins with a somewhat complex review of the current thinking on the genetic basis for cellular senescence and immortality, the latter particularly in relation to the immortalization of keratinocytes. The bulk of the book (13 chapters) contains protocols for producing immortalized cells from a variety of (mostly human) sources including hepatocytes, astrocytes, keratinocytes and glial cells. A further chapter details methods available for mapping human senescence genes. Each of the 'methods' chapters contains useful introductory material, though the amount varies considerably. The protocols themselves are presented in a clear and straightforward manner, which should be relatively easy to follow, and there is surprisingly little overlap between chapters. Each chapter is fully referenced, though it is claimed that the detailed protocols obviate the need for primary literature sources. In addition, there is a very useful and timely chapter on safety aspects of working with immortalized cells.

Culture of Immortalised Cells assumes a good working knowledge of tissue culture and molecular biological techniques and therefore cannot really be aimed at undergraduates. Given the nature of the work and the high level at which it is written, it will be invaluable for research personnel in tissue culture laboratories where cell immortalization is being contemplated. Of particular value is the variety of cell sources covered, allowing a good overview of what has been achieved so far, and the comprehensive information on methods, reagents and suppliers. In summary, an excellent source of information in a highly specialized field.

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Organotypic protocols

Epithelial Cell Culture: A Practical Approach

edited by A. J. Shaw, Oxford University Press, 1996. UK£27.50 (238 pages) ISBN 0 19 96 3572 2 (paperback) UK£55.00 ISBN 0 19 963573 0 (hardback)

Call me old-fashioned but I always like to start reading a book at the beginning, with the preface. In this case this was a very good place to start as Dr Shaw's aims for the book are spelled out quite concisely:

- to provide detailed protocols for *modelling* [my emphasis] different epithelia *in vitro* using primary cultures or cell lines;
- to provide detailed protocols for morphologically, biochemically and functionally characterizing these models in relation to their *in vivo* counterparts; and
- to outline research applications to which these cultures can be applied.

The clue to the real nature of this book comes earlier in the preface where Dr Shaw points out that a common theme is the culture of epithelia on porous substrates, so it should really be called: 'Organotypic Culture: A Practical Approach' – but then it would probably not sell well. This would be a shame because Dr Shaw has put together a fine collection of chapters that more than adequately meet his aims.

Chapter 1 serves as an excellent introduction to the nature of

epithelia (although I am sceptical about Dr Shaw's efforts to include endothelial cells under this banner), and, whilst it does not contain any protocols *per se*, does serve as a useful reference source for the coming chapters. One item that might usefully have been included here would be a discussion of how primary biopsy material, particularly human (although handling bovine brain for the protocols in Appendix 2 might presently be considered hazardous too!), may be obtained and handled. There are ethical and safety issues here that are not adequately addressed in any of the remaining chapters. I also found it slightly disappointing that no reference to systems for culture of breast epithelium is made here (or, indeed, anywhere else before page 136). These minor criticisms aside, Chapter 1 does whet the appetite for what follows, and what follows are eight chapters and appendices describing the culture, characterization and uses of human keratinocytes, canine kidney epithelial cells, human and rat hepatocytes, a human intestinal epithelial cell line, guinea pig endometrial cells, human endothelial cells, rat Sertoli cells and bovine brain microvascular endothelial cells. Each of these chapters contains the expected detailed and well illustrated protocols, each is well referenced and, with one exception, well introduced and discussed. The exception is Chapter 4 on xenobiotic metabolism, in which the authors launch into the protocols without adequately introducing the subject. Nowhere can the uninitiated find a description of the nature of Phase I and II reactions, or what they are important for, or why one should wish to assess them in hepatocytes.

It is clear that this volume is targeted at those scientists who wish to study toxicology, pharmacology and pharmaceutics *in vitro*. It will therefore appeal to those engaged in industrial applications as well as those engaged in more basic research. It is also clearly targeted predominantly at the young – who else could decipher the tiny font used to list the ingredients at the top of each protocol?

Other reviewers of books in this series have carped at the lack of telephone or fax numbers for the suppliers listed in the appendices. I am not convinced these would be useful as there are often many contact numbers and, in the UK at least, these are prone to change with monotonous regularity. However, it might be useful to include World Wide Web site addresses as a single point of reference in future.

My recommendation? If you are interested in organotypic cultures of epithelial cells, buy this book and it will serve you well. If you are interested in more basic epithelial cell culture, others might be of more use.

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Cutting proteins down to size

Industrial Enzymology (2nd edn)

edited by T. Godfrey and S. West, Macmillan, 1996. UK£110.00 (xiv + 609 pages) ISBN 1 56159 163 7

Since the first edition of this book appeared in 1983, there have been many significant developments in enzymology and related fields. The editors have, therefore, updated the previous edition and, in this volume, provide a comprehensive account of current practice in industrial enzymology, with a topical focus on environmental aspects, concerns with food safety and newer applications such as biosensor development. The book opens with a brief introduction to the subject, emphasizing commercial aspects such as current/projected markets for industrial enzymes and factors important in selecting enzymes for particular processes. The bulk of the book (almost 400 pages) focuses on detailed descriptions of applications of enzymes to some 22 industrial sectors, ranging from biosensors to textiles. It concludes with chapters on legislative, toxicological and safety aspects of the topic and with a description of applied enzyme kinetics. In addition, there is a lot of compiled information provided as tables or appendices, such as pH/temperature properties of industrial enzymes, names and addresses of commercial suppliers of enzymes and other products and a guide to the use of enzymes in the more common industrial areas. This is a valuable addition to the literature of applied enzymology.

In general, I found the treatment to be comprehensive and readable. The editors have done a fine job in producing a multi-author volume with a high degree of internal consistency in terms of style and content. With the exception of a small number of annoying typographical errors in parts of the book, it is produced to quite a high standard with frequent use of tables, drawings and chemical structures. The book is not without its flaws, though. In the light of developments in protein expression systems and downstream processing of proteins in the last 13 years, I was surprised that no coverage was given to the actual industrial production of enzymes for the industries described. This could have provided an important unifying strand through the book and would have given the opportunity of exploring the varying levels of purity/activity required for different applications (for example in diagnostic application compared, say, to use in a food-related process). Several contributors allude to variation in properties of industrial

enzymes from different batches or different suppliers so this, in my opinion, represents an important omission. I also felt that the chapter on practical applied kinetics, while sound in itself, could have been significantly updated by a description of non-linear regression and other computer fitting programmes for analysis of kinetic data. These weaknesses of emphasis are not by any means fatal to the book but could, perhaps, be addressed in future editions.

This volume would be of interest to a very wide audience, including biochemists/microbiologists involved in industries in which enzymes are important, undergraduate/graduate students of disciplines such as chemical engineering and biotechnology and research scientists interested in possible commercial applications of their work. I found it a well-written, clear and interesting description of this important area.

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Viruses as tools for human gene transfer

Viruses in Human Gene Therapy

edited by Jean-Michel H. Vos, Chapman & Hall, 1995. UK£39.00 (xiii + 213 pages) ISBN 0 4126 3160 1

Gene therapy has enormous potential to treat both heritable and acquired genetic diseases, by introducing genetic material into specific cells of an affected patient, to replace or augment defective genes. As a means of introducing genetic material into human cells, scientists are exploiting the strategies of certain viruses that have naturally evolved as experts in human gene transfer. *Viruses in Human Gene Therapy* reviews six major virus families currently under development as gene transfer vehicles. There are chapters describing viral vector systems already employed in early Phase I/II clinical trials, such as recombinant retroviruses, adenoviruses and poxviruses. In addition, there are chapters describing viral systems showing potential as gene delivery systems, such as adeno-associated, herpes and hepatitis delta viruses, including a chapter describing a combination of physical and viral-based gene transfer. The chapters are clearly written by world leaders in viral gene transfer technology. Each chapter provides a comprehensive review of the molecular biology of each virus family, including detailed descriptions of the genetic engineering of recombinant viruses. Also presented are numerous examples of the application of recombinant viruses carrying therapeutic DNA *in vivo*, mainly in animal models. Contributors address issues of safety and the disadvantages associated with each viral system, with the exception of the adenovirus chapter by Perricaudet and Stratford-Perricaudet. They describe the capacity of recombinant replication deficient adenovirus for long-term gene transfer in rodents but there was a marked lack of information about the significant immune response against recombinant adenoviruses.

Other gene transfer systems are presented in a candid manner. As an alternative to recombinant viral vectors, Curiel discusses adenovirus-polylysine-DNA complexes as a non-viral gene transfer mechanism that exploits the lysosome disruption ability of the adenovirus virion, without expression from the viral genome. Cox, Gettig and Paoletti present a concise chapter on poxviruses as viral vectors for the transient expression of immunologically relevant proteins for immunization. Herpes virus is being developed as a viral vector capable of latent non-neurovirulent and non-cytopathic gene transfer, as summarized by Vos. The hepatitis delta virus, which naturally infects the liver *in vivo*, has recently gained favour as a viral vector with the potential to deliver small RNA sequences such as ribozymes and antisense RNA to the liver, although, as discussed by Netter, Lazinski and Taylor, the investigations are preliminary.

The book contains two articles about integrating viral systems. Retroviruses and adeno-associated virus are both able to integrate into the host cell genome, establishing the potential for long-term gene expression. The technology of retrovirus-mediated gene transfer is succinctly reviewed by Morgan, who also describes some of the human clinical trials that have used this technology to date. Samulski provides a detailed account of adeno-associated virus (AAV) vectors. Only examples of *in vitro* transduction of recombinant AAV are presented, as *in vivo* studies have been hampered by the generation of low titres of recombinant virus due to inferior packaging systems.

In conclusion, the book is a valuable resource of information on six major viral gene transfer strategies. Each chapter provides comprehensive reviews and descriptions of particular viral systems, complete with detailed bibliographies. The only unavoidable criticism is that the field of gene therapy is a rapidly advancing one, and some of the data presented in the book are already dated. However, the book does function as an excellent introduction to the use of viruses in gene transfer.

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Genetics on the move into natural-product chemistry

Meeting report

The application of enzymes and multienzymes in natural-product chemistry is at an exciting stage. The total enzymatic *in vitro* synthesis of the vitamin B12 precursor hydrogenobyrinic acid, via its aerobic pathway, by 12 enzymes expressed in *Escherichia coli* with a 70% yield was referred to as the thrill of an overnight experiment by Ian Scott (Texas A & M University, TX, USA) at a recent meeting on natural-product-biosynthesis★. His lecture was one of many highlights.

Nonribosomal peptide synthesis

The nonribosomal peptide forming system was a major focus of the meeting, which was attended by 130 researchers†. Horst Kleinkauf [Technical University (TU), Berlin, Germany] summarized the main features of this modular system, which can direct the formation of diverse peptides, such as penicillin, gramicidin S, Ca^{2+}-dependent lipopeptide antibacterials (e.g. CDA or daptomycin), immunomodulators (e.g. cyclosporin), chromopeptides (e.g. actinomycin), antihelminthic depsipeptides, the herbicide bialaphos and the peptide moiety of ergot alkaloids. The key pieces of the nonribosomal code are amino acid selecting- and activating-modules, which structurally resemble acyl-CoA synthetases and insect luciferases, but are clearly distinct from aminoacyl-tRNA synthetases[1]. During this meeting, structural details of more than 30 such modules were reported. The activating module has been dissected into two subdomains, which by conformational change combine in the formation and protection of the aminoacyladenylate (Ralf Dieckmann, Maja Pavela-Vrancic, Hans von Döhren and Horst Kleinkauf, TU, Berlin, Germany). Studies of hybrids indicate that about 300 amino acids of the modules are involved in amino acid selection (Frank Bernhard and Andrea Elsner, Free University, Berlin, Germany). Activated amino acids then serve as intermediates in the thiotemplate mechanism[1], but they have to be transferred to an acyl carrier protein or domain.

Acyl and peptidyl carrier proteins

The role of tRNA in nonribosomal peptide synthesis is played by an acyl carrier protein structurally identical to its counterpart in polyketide systems. Its key component is the CoA-constituent 4′-phosphopantetheine added by post-translational modification. A thrilling result reported by Mohamed Marahiel (University of Marburg, Germany) and Peter Zuber (Louisiana State University, Shreveport, LA, USA) was the final identification of a notorious protein that is repeatedly found in various biosynthetic clusters as the cofactor-adding enzyme, functioning in this post-translational modification of peptide synthetases and presumably in polyketide synthases by CoA. This result has to be credited to Chris Walsh's collaborators, including Ralph Lambalot (Harvard University, Cambridge, MA, USA), who by careful analysis of sequence data uncovered at least some structural similarity of proteins like sfp or gsp with the *E. coli holo*-ACP synthase reported last year[2]. This achievement, hopefully, will lead to the overexpression of fully functional synthetases and synthases and their exploitation in condensing unit operations as precursors to peptides and polyketides. The mode of attachment of the cofactor has finally been analysed in full by Joachim Vater, Torsten Stein and collaborators (TU, Berlin, Germany) who employed advanced mass spectrometric techniques. This group also determined the mass of gramicidin S synthetase 2 by the MALDI technique (matrix assisted laser desorption mass spectrometry) as 512 kDa; this represents the current record for proteins but other giant enzymes like rapamycin synthases (660, 900 and 1070 kDa) or cyclosporin synthetase may follow (1.7 Mda). The latter is the largest enzyme known, and contains 11 peptidyl carrier domains.

The nonribosomal code

Efforts are being made to manipulate multienzyme templates by module replacements. As the number of possible constituents known from product structures is already around 500 L-, D-, β, γ and δ amino acids, as well as imino and hydroxy acids, the prospects for synthetic reactions are impressive. The replacement of activation/carrier module tandems in the surfactin system has been carried out by Mohamed Marahiel and colleagues, and new peptides have been generated, replacing one of the activation domains with bacterial and fungal domains by repeated recombination in *Bacillus subtilis*. Attempts to select for module replacements in *Streptomyces* model systems encoding lipopeptide antibacterials have been reported by Thomas Hosted from Dick Baltz's group at Eli Lilly (Indianapolis, IN, USA). In a comparative analysis of enniatin synthetase activation modules with specificities for different branched chain amino acids, Anke Doller, Angela Haese and Rainer Zocher (TU, Berlin, Germany) have identified the very subtle sequence changes responsible for side chain selection. Geoff Turner's group (University of Sheffield, Sheffield, UK) reported the construction of hybrid tripeptide synthetases, using the ACV synthetase gene and replacing modules by direct selection in *A. nidulans*. By point mutation in the terminating thioesterase domain, the formation of the LLL-ACV tripeptide was triggered, which is the first example of a configurational change achieved in a biosynthetic system (Wibke Kallow, TU, Berlin, Germany).

In vitro systems

The *in vitro* exploitation of enzyme systems is just beginning but has already led to surprising results. Besides the already reported synthesis of dipeptides with the ACV synthetase system[3], Mirko Glinski, Matthias Walter and Rainer Zocher (TU, Berlin) have reported the formation of a variety of cyclodipeptides with the cyclosporin synthetase. These data imply the possibility of intermediate starts, in the analogy to the classical translation system employing polyU as mRNA, and forcing the aminoacyl-component in the peptidyl site. Of considerably more importance for the natural product chemist is the replacement of branched-chain aliphatic amino acids by serine and threonine in a

★The symposium 'Enzymology of Biosynthesis of Natural Products', was organized by the special research group Integrated Enzyme Systems, and was held in Berlin, Germany, 22–25 September 1996.

†Copies of the proceedings may be ordered from the author.

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PII: S0167-7799(97)01010-X

cyclodepsipeptide system, facilitating their chemical modification in the development of antihelminthics (Rainer Zocher and collaborators; TU and Bayer AG, Germany).

System identification

The available tools of core sequence-directed cloning of peptide synthetases, their detection by specific antibodies, or cluster analysis together with amino acid activation assays permit the identification of new biosynthetic systems. New details were reported of peptide synthetase genes of tyrocidine (*Bacillus brevis*: Henning Mootz and Mohamed Marahiel; Marburg University, Marburg, Germany), fengycin (*B. subtilis*: Shih-Tung Liu and colleagues; Chang-Gung College, Taoyuan, Taiwan), actinomycin (*Streptomyces chrysomallus*: Frank Schauwecker, Frank Pfennig and Ulrich Keller; TU, Berlin), CDA-peptide (*S. coelicolor*: Colin Smith and associates; Manchester Institute of Science and Technology, Manchester, UK), nosiheptide (*S. actuosis*) and thiostreptone (*S. laurentii*: Heinz Floss and colleagues; University of Washington, Seattle, WA, USA), the nikkomycin pathway (*S. tendae*: Bettina Lauer, Christine Bruntner and Christiane Bormann; University of Tübingen, Tübingen, Germany), phosphinotricine (*S. viridochromogenes*: Wolfgang Wohlleben and colleagues; University of Tübingen), lysobactin (*Lysobacter* sp.: Frank Bernhard and colleagues; Free University, Berlin) and cyanobacterial peptides (both *Microcystis* and *Anabaena*: Thomas Börner, Brett Neilan, Elke Dittmann and colleagues from the Humboldt University, Berlin, and Kaarina Sivonen and Leo Rouhiainen, University of Helsinki, Finland). Cyanobacteria and the dalbaheptide producer *Amycolatopsis mediterranei* (Wolfgang Wohlleben and colleagues) have been shown to contain multiple peptide synthetase genes. Peptide synthetase genes not yet characterized were also reported from *B. subtilis* and *Cylindrotrichum oligospermum* (Frank Bernhard) and *Pseudomonas fluorescens* (Narayanan Rajendran; Anna University, Madras, India; and Mohamed Marahiel). A particular success has been the identification by targeted gene disruption of microcystin biosynthetic genes, presumably encoded by a large plasmid (Elke Dittmann and Thomas Börner).

Progress has been reported in the enzymology of gramicidin S (Kazuko Hori; Hyogo College, Hyogo, Japan) iturin (Francoise Besson; University of Lyon, France), aromatic and heteroaromatic peptidolactones and ergot peptides, as well as phosphinothricin (Ullrich Keller and colleagues), daptomycin-type peptides from *S. fradiae* and *S. roseosporus* (Petra Weßels and Hans von Döhren; TU, Berlin), and ACV synthetase (Juan Martin and colleagues; University of Leon, Spain). The latter system has been used to finally confirm the energy requirement of one mole of ATP per peptide bond for the nonribosomal system (Wibke Kallow and Hans von Döhren).

Cooperating enzyme systems

The interactions of peptide-forming systems with polyketide biosynthetic systems have been largely ignored, although the cooperation of these systems in lipopeptide synthesis is obvious. The same is true for interactions with fatty-acid-forming systems, which provide starter molecules for polyketide synthases of both type I and II. The polyketide session was opened by Peter Stuible from Eckehard Schweizer's group (University of Erlangen, Erlangen, Germany) who described two fully-integrated fatty acid synthases from *Brevibacterium ammoniagenes* with different product profiles. Evidence has been accumulating that certain of these synthases catalyse their own post-translational modifications with 4′-phosphopantetheine, but definite proof has yet to be obtained. Alexander Steinbüchel (University of Münster, Münster, Germany) then reviewed the current state of analysis of polyhydroxyalkanoate-forming systems; polyhydroxyalkanoates represent a special type of polyketide homopolymer, forming globule structures surrounded by specific proteins. More than 100 constituents have been found in these versatile biopolymers.

David Hopwood (John Innes Institute, Norwich, UK) gave an overview on the state of mix-and-match experiments in aromatic polyketide systems, currently viewed as the beginning of combinatorial biosynthesis. These type II systems are far from being understood because of the lack of structural data but have nevertheless yielded impressive results. Bill Strohl and Vineet Rajgarhia (Ohio State University, Columbus, OH, USA) and Dick Hutchinson (University of Wisconsin, Madison, WI, USA) discussed functions and interactions in the daunorubicin, tetracenomycin and related polyketide systems with special reference to starter molecules and cyclization modes. Functions assigned in so-called minimal polyketides synthase systems may be modulated by additional proteins. Late modifications of polyketides were then addressed by Jürgen Rohr (University of Göttingen, Göttingen, Germany), illustrating the potential of oxygenation and glycosylation in the design of new products and producer strains. More type II clusters have been made available: mithramycin (Felipe Lombo, José Salas and colleagues, University of Oviedo, Oviedo, Spain), nogalamycin (Kristiina Ylinonko, University of Turku, Turku, Finland), and avilamycin (Sibylle Gaisser, Sigrid Stockert, Axel Trefzer and Andreas Bechthold, University of Tübingen); these will permit more natural combinatorial approaches.

The type I polyketide systems now have a clear lead in extent of manipulation, and this is mainly the achievement of Peter Leadlay and his colleagues (University of Cambridge, Cambridge, UK). Following the initial work of inactivation of single modification functions in the erythromycin system, they have now not only demonstrated repositioning of the termination module but also the exchange of specificity (propionate for acetate) and the exchange of the starter unit (substituting the avermectin starter into the erythromycin system). These spectacular achievements were presented at the meeting together with a detailed model of a polyketide system involving dimer formation and domain interactions. In addition, the sequencing of the rapamycin (Peter Leadlay and colleagues) and immunomycin (Haideh Motamedi, Merck Sharp & Dohme, Rahway, NJ, USA) biosynthetic clusters links the peptide and polyketide pathways. The Merck group provided new analogs by targeted disruption of genes within the cluster, encoding oxygenase and methyltransferase functions (Ali Shafiee and colleagues). Another mixed pathway combining a polyketide synthase and a peptide synthetase has led to the phytotoxin coronatin. The respective genes have been analysed by Carol Bender's group at the University of Oklahoma

(Stillwater, OK, USA), and the plasmid-encoded cluster, which is regulated, depending on the temperature, by a two-component system was discussed by her former co-worker Matthias Ullrich (now at the Max-Planck-Institute, Marburg, Germany).

Plant metabolites

A third type of polyketide-forming enzymes, a superfamily so far restricted to plants was introduced by Joachim Schröder (University of Freiburg, Freiburg, Germany). These form chalcones, stilbenes and related compounds, such as acridones or hop bitter acids (Karin Zuurbier, Robert Verpoorte and colleagues; Leiden University, Leiden, The Netherlands). Other enzyme systems of plant origin under discussion included the berberine bridge enzyme that transforms reticuline into scoulerine in the biosynthesis of alkaloids, and an oxygenase catalysing berbamunine-formation by phenol coupling (Toni Kutchan; University of Munich, Munich, Germany). The respective pathway enzymes are not clustered but have been made accessible by expression in yeast or baculovirus-infected insect cells. Progress in isoprenoid enzymology was considered by Thomas Bach (University of Strasbourg, Strasbourg, France) and Jörg Bohlmann, representing Rodney Croteau's lab (Washington State University, Pullman, WA, USA). Bach described the cloning of plant 3-hydroxy-3-methylglutaryl-CoA reductases and acetoacetyl-CoA thiolase by complementation of yeast mutants. He also addressed the possible role of compartmentation in the formation of specific products. Bohlmann depicted model systems of terpene synthases, focusing on the mechanical defence response of the grand fir. Terpene synthases have been successfully expressed in *E. coli*, and their functional specializations are now under investigation. An emerging theme in terpenoid-transforming enzymes in plants has been the involvement of steroids in developmental processes, similar to steroid hormones in animal systems; Annette Kauschmann (Institute of Genebiological Research, Berlin), Martin Luckner, Peter Lindemann and colleagues (University of Halle, Halle, Germany) and Robert van der Heijden and colleagues (Leiden University, Leiden, The Netherlands).

Regulatory networks

To illustrate the complex events of regulation of secondary metabolites the two best studied systems have been selected, surfactin (*B. subtilis*) and penicillin (*A. nidulans* and *Penicillium chrysogenum*). Surfactin had been suspected as a key molecule in competence development but it was only last year that the peptide comS emerged as a small gene within a peptide synthetase gene, in opposite orientation. Peter Zuber described the competence network, now comprising over 30 genes. Leendert Hamoen (Groningen University, Groningen, The Netherlands) reported an elegant *in vitro* analysis on the interactions of ComS with CmK, ClpC and MecA carried out in David Dubnau's lab (Public Health Research Institute, New York, NY, USA) in collaboration with Kursad Turgay and Jeanette Hahn, which again demonstrated nicely the complexity of the interaction of multicomponent systems, the future theme of this field.

The eukaryotic systems of penicillin production opened up a complex field of transcription factors. Axel Brakhage (University of Munich, Munich, Germany) described the identification of specific binding sequences within the bidirectional penicillin promoter and their *in vivo* confirmations. Juan Martin compared β-lactam biosynthetic clusters of various prokaryote and eukaryotes and their relation to precursor biosynthesis. Of special interest has been the extensive cluster amplification in production strains of *P. chrysogenum*, and the identification of possible hot spots of recombination involved in such processes.

Ribosomal translation

In a final tribute to the ribosomal system, Paul Schimmel (MIT, Cambridge, MA, USA) and Knud Nierhaus (Max-Planck-Institute of Molecular Genetics, Berlin, Germany) discussed the role of RNA structures in amino acid recognition and the peptidyltransferase centre. These fundamental contributions have emerged only in the last few years. Possible evolutionary connections between the translational system and the programmed enzyme systems that perform similar directed condensations are now considered less significant in a direct structural comparison. However, spatial structures are expected to become known in the near future and may provide new insights.

Conclusion

In recent years much effort has been devoted to characterizing genes and the respective proteins forming natural products. This work has revealed an impressive enzymology and at the same time provided us with some insight into the molecular organization of reaction sequences leading to complex molecules. First attempts are now being made to redirect enzyme systems to generate new compounds. This opens up the possibility of exploiting nature's own combinatorial devices, making use of an elegant chemistry that also permits the production of compounds by biotechnological means.

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Meeting report

If you've got it, flaunt it – rapid screening for microbial biocatalysts

The main approaches to drug design are either 'rational', based on a knowledge of the 3D (though usually static) structure of the receptor of interest, or more usually by empirical test receptor binding, exploiting advanced robotic and data handling assay systems and often testing against several potential disease targets. The growth in both natural-product-based and purely synthetic combinatorial compound libraries is thus driving a High Throughput Screening (HTS) revolution in drug discovery that now sees large pharmaceutical companies able to test as many as 50 000 samples per day for biological activity, or 30 times as many as they could just three years ago.

Similarly, the development of improved biocatalysts using microbial strains and enzymes traditionally relies on screening large numbers of entities for optimum activity and then going into strain development programmes and modifying fermentation conditions to increase titre or activity. Thus, the emergence of exciting new technologies, driven by advances in HTS for drug discovery, has resulted in the recognition of a need for screening ever increased numbers of samples for biocatalytic activity, through automation, miniaturization, rapid single-step assays, and multi-assays, providing for greater product diversity and exploiting economies of scale to limit cost.

The challenge to industrial and academic researchers in developing technologies to meet these needs was the theme of a recent symposium* that brought together scientists from many of the established pharmaceutical companies, the often small but rapidly-evolving biotechnology screening and niche product development companies, and consultants and academic researchers working at the forefront of screening technology. Unusually, the majority of participants came from industry.

The advantages of biocatalysts, i.e. of using biotransformations compared with chemical synthesis, are well known, and include the shortening of synthetic pathways, the use of mild reaction conditions and inexpensive substrates, the potential to generate compounds of a given optical or regiospecificity in high yield and to use a wide substrate range (Klaus Kieslich; GBF, Braunschweig, Germany). Catalogues of known bioconversions are now available on a searchable CD-ROM. Although the use of biocatalysts has distinct attractions, random searching for novel microorganisms suitable for biotransformations is a time-consuming and laborious process. Thus a number of enrichment techniques have been developed to circumvent this, including those based on the isolation of microorganisms from environments correlated with the educts, such as alkane degraders from oilfields, and intuitive selection of strains from culture collections based on a priori knowledge (e.g. taxonomic relationships with strains previously investigated for similar phenomena). The effectiveness of such developed strains could be very great, with the *Rhodococcus rhodochrous* J-1 nitrile hydratase system yielding no less than 656 $g\,l^{-1}$ of acrylamide from acrylonitrile in a 10 000 $t\,y^{-1}$ process (Hideaki Yamada; Toyama Prefectural University, Japan).

Even where potential biotransformation systems have been identified, there is still much room for improvement. Screening ~300 commercial enzyme preparations would typically require 2–5 g of the substrate and where the products are analysed by HPLC or GC this takes, at least 8 min per sample, notwithstanding that one would probably need to try several temperatures, solvents and incubation times (David R. Dodds; Schering-Plough, Kenilworth, NJ, USA). Microbial screening requires even more substrate and is additionally disadvantaged by the normal requirement for an aqueous (or at least biocompatible) environment; as a result primary selection is typically based on following up those strains exhibiting >5% of the desired bioconversion. Substrates derived from upstream processing that is optimized merely for their production may include contaminants inhibitory to the biocatalytic process under development, whilst the use of model chromogenic or fluorogenic substrates is often inconvenient and inappropriate since the biocatalyst must act on the actual substrate of interest. As large-scale processing is the most difficult area of biotechnology in which to motivate change, reaction conditions should accommodate the likely scale-up conditions as closely as possible, and for economic reasons the analytical methods should be direct, quantitative and minimize sample handling. Different parameters interact in complex ways, for example a solvent that is best at one temperature may be much worse at another temperature; only the most up-to-date, hierarchical experimental designs will pick this up.

Born to be wild – tapping into microbial diversity

The use of modern molecular methods such as PCR, nucleotide sequencing and rRNA analyses demonstrates unequivocally that only a minuscule number (probably less than 0.1%) of microbial species have as yet been cultured, which obviously leaves a substantial untapped resource for generating novel biocatalysts (Erko Stackebrandt; DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany). The construction of phylogenetic trees provides a framework for establishing relatedness and the diversity of novel strains, to seek to maximize exploration of the biodiversity space. The need to learn how to culture uncultured microorganisms is now a high priority, and this may well be assisted by analysing their molecular taxonomy. However, there are as yet few rules governing the distribution of biocatalytic activities among the major taxa.

The search for novel biocatalysts might therefore be based on a focused screen using known enzyme producers or a more extensive random screen with the emphasis on making use of such biodiversity (Graham Byng; Panlabs Inc., Bothell, WA, USA). Increasing diversity typically involves the targeting of different habitats, selecting single representative strains chosen

*The meeting 'New Frontiers in Screening for Microbial Biocatalysts' was organized by W. J. J. van den Tweel, J. A. M. de Bont and C. P. van der Beek, under the auspices of the Applied Biocatalysis and Microbial Physiology Working Parties of The European Federation of Biotechnology, and was held in Ede, The Netherlands, 15–18 December 1996.

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PII: S0167-7799(97)01009-3

by morphological comparison and employing different fermentation media and growth conditions. In an example study of microorganisms isolated from *Spartina alterniflora* cord grass in a salt marsh habitat, visual inspection and fatty acid methyl ester fingerprinting were used to distinguish 70 groupings in ~600 isolated strains. Dereplication at this point is considered essential to reduce the unnecessary workload that would ensue from having duplicate strains in downstream analysis.

Indeed, the importance of harnessing microbial diversity for accelerating drug discovery comes to the fore when coupled with the latest assay systems and sample presentation techniques. Modifying the growth conditions to mimic those in nature more closely would help persuade such organisms to make additional, and more interesting, secondary metabolites, and this proved to be true of biocatalysts too (Neil Porter; Biodiversity Ltd, Uxbridge, UK); increasing the efficiency of screening larger numbers of samples by using 384-well microtitre plates instead of the 96-well format, using homogeneous assays with just one process step in the assay and exploiting data-rich methods such as LC-MS were also important ways forward.

Sharing the burden – robotics and automation

The large step-changes in sample screening numbers require a concomitant increase in the generation of samples, which is unlikely to be met by traditional methods. Manually intensive methods of isolating and screening microbial strains for novel or desired catalytic activities or products have been the mainstay of the industry for many years, typically involving enrichment from a heterogeneous population (often derived from soil), and assaying for activity in culture tubes. However, after the initial enrichment and colony selection these methods tend to be repetitive and can be automated to reduce the resources in manpower and time needed to find a biocatalyst. Modern robotic systems can localize colonies (using image analysis), subculture and inoculate them into liquid culture, and can subsequently perform the liquid handling tasks required for assay of the broths for activity. Typically 10–100 000 strains may be assayed for a new biocatalytic activity, and this combination allowed the screening of 1000 strains per week (Olgé Favre-Bulle; Rhône-Poulenc Industrialisation, St Fons, France). Even using object-oriented software, scheduling and exception handling are particularly important tasks for such robotic applications (Alain Donzel; Scitec, Lausanne, Switzerland).

Expression cloning

Although the classical approaches of microbial screening for novel enzymes may be sufficient to produce a biocatalyst with high activity, molecular biological techniques can be used to increase yields by expression cloning. Isolation of the enzyme by cloning serves a number of purposes, including separation from a complex mixture in the original host and making it possible to manipulate the gene for the enzyme of interest (and its regulation). Indeed, users are now beginning to demand monocomponent enzyme preparations (Henrik Dalbøge; Novo Nordisk A/S, Bagsværd, Denmark). More than 200 different enzyme genes from fungi of interest have been cloned in *Saccharomyces cerevisiae* cDNA libraries by detecting expression with azurin-, PNP- and MUF-linked substrates. In this case enzyme expression could be increased by transferring the genes into a highly-transformable fungal host (*Aspergillus*) under the control of selected promoters. Similarly, screening for esterases using chromogenic or fluorogenic esters allowed the genes coding for ten esterases to be obtained from four different bacterial genera (Helmut Schwab; University of Technology, Graz, Austria). The cloning approach could be taken a step further by gathering published information about similar nucleotide sequences and using this to produce PCR primers that allow the isolation of similar enzymes (with perhaps slightly different properties) from non-culturable strains.

Molecular tagging

The ideal assay system for HTS would be a single manipulation (i.e. sample delivery) performed in a single well; it should be easily automated and preferably allow for the screening of a number of different activities simultaneously. One of the main challenges of isolating clones containing an enzyme activity of interest is developing simple assays sensitive enough to determine any level of activity that may represent an enzyme with optimal activity against a related substrate. Novel colorimetric, luminescence and fluorescence methods have been developed as homogeneous assays applicable to HTS, with automated 'multiplex' (typically 10–20 compounds per well) processing, including variations on the theme of lanthanide measurement based on Dissociation Enhanced Lanthanide Fluoroimmunoassay (DELFIA) and Homogenous Time-resolved Fluorescence (HTRF) (Stuart Webb; Wallac Oy, Turku, Finland). HTS multiplexing can be taken a step further by using a multiple label immunoassay, with as many as four different lanthanides to assay for different enzymes of interest. Separation-free/solution format HTRF screening can provide 2000 samples per hour throughput (Al Kolb; Packard Instrument Company, Meriden, CT, USA), although many advances in membrane filtration materials are making separation much less of an issue (Robin Hood; FSM Technologies, Clydebank, UK).

Many enzymes are difficult to screen because of their poor activity in aqueous environments. Thus a useful development was a series of new commercial substrates for lipase assays that are compatible with a range of reaction media. These consist of alkyldiacyl glycerols, containing a pyrene fluorophore and a trinitro phenylamino residue as quencher, that increase in fluorescence intensity upon hydrolysis. Assaying a range of these fluorescent derivatives allows lipases with different stereopreferences to be isolated (Albin Hermetter; University of Technology, Graz, Austria).

Getting real – microbial screening in practice

Lithium therapy, a major treatment for manic depression, is thought to act mainly through inhibiting *myo*-inositol monophosphatase (IMPase) [Axel J. Glanzhorn; Synthélabo Biomoléculaire (formerly Marion Merrell Research Institute), Strasbourg, France]. Natural products from 12 000 microbial fermentation broths and 30 000 synthetic compounds were screened with a robotic, automated, 96-well microtitre plate assay based on inhibition of IMPase activity using an ammonium molybdate/malachite green assay for measurement of inorganic phosphate. This resulted in the eventual isolation of one compound that acted as an irreversible inhibitor of IMPase.

Despite all of the systematic progress that will undoubtedly be made in developing processes by straightforward automated HTS, it is refreshing to note that some important breakthroughs should still come about through serendipity. Success in screening for microbial strains capable of generating artificial nucleoside analogues (some of which have been developed as anti-HIV agents) was initially achieved during an extensive screening programme by an accidental increase of the incubation temperature of *Enterobacter aerogenes* biosynthesizing adenine arabinoside. Investigation of the thermal characteristics of the process, which was prompted by this fortuitous discovery, revealed that the higher temperature (~50°C) was essential for reducing a deaminase activity in favour of the desired nucleoside hydrolysis (Takashi Utagawa; Ajinomoto Co. Inc., Japan). The use of higher temperatures during enzyme purification allowed use of the biocatalyst to be extended to the preparation of many other purine arabinosides. In general, a systematic approach based on enrichment or acclimation to obtain an activity, subsequent enzyme isolation and purification, followed by N-terminal sequencing and cloning, provided a rational approach that reproducibly delivered the goods (Yasuhisa Asano; Toyama Prefectural University, Japan).

Combining biodiversity and focused approaches

There are many advantages of utilizing microorganisms (e.g. thermophiles, halophiles) from so-called extreme habitats, where one may expect to discover unusual new enzymes with desirable properties (Jay M. Short; Recombinant Biocatalysis Inc., Sharon Hill, PA, USA). A novel approach to biocatalyst discovery and development, which is already being exploited commercially, involves preparing novel enzymes from these sources by elimination of the need to culture the microorganism. As discussed above, the vast majority of microorganisms have yet to be cultured or may be non-culturable under known conditions. Therefore, any method of using the biocatalytic potential of such non-cultured cells that eliminates the need for this step is of great value. This is achieved by isolating nucleic acids directly from environmental samples or primary enrichment cultures and constructing gene libraries for HTS for particular enzyme activities. This 'Biopanning' of biodiverse environmental libraries provides cloned genetic material which can be further processed by directed evolution, for example by gene-shuffling, to combine the best properties of enzymes from a variety of sources.

Single cell selection methods

As a specific example of the development of biocatalysts for commercial processes, that for preparing the valuable intermediate (*R*)-2-(4-hydroxyphenoxy)propionic acid (HPOPS) is illustrative (Bernard Hauer; BASF AG, Ludwigshafen, Germany). Three strains were selected from a screening programme which analysed many thousands for the ability to hydroxylate the precursor POPS. The fungus *Beauveria bassiana* was chosen for strain improvement; by selection for POPS tolerance and UV mutagenesis the yield increased from $0.5\,g\,l^{-1}$ to $2\,gl^{-1}$ and by chemical mutagenesis to $7\,g\,l^{-1}$. Flow cytometric cell sorting was used to isolate elongated spores which correlated with a higher yield of HPOPS when cultivated and tested for hydroxylation of POPS in a microtitre plate assay. The current process operates at the 100 000 l scale.

Single cell selection is fundamental to the methods currently being used by the evolutionary biotechnology company Evotec (Björn F. Lindemann, Evotec Biosystems, Hamburg, Germany). Evolutionary strategies for efficient engineering of biocatalysts *in vitro* are being developed by generating genetic diversity through random mutagenesis; highly active variants are then located by bacterial display under physiological conditions, or by using the Fluorescent Enzyme Bead Assay System (FEBAS) where there is free-diffusion of the assay substrate but localization of the fluorescent product. In a miniaturized screening system consisting of 8000 compartments contained within a 4 inch wafer, volumes of less than 750 nl can be screened in drops of 0.5–3.5 nl volume dispensed at a rate of $3000\,s^{-1}$. Cells or beads can thus be assayed individually or in small groups using fluorescence correlation spectroscopy and confocal fluorescence scanning; cells presenting the product of interest can be identified by Confocal Nanospectroscopic Scanning (CNS) and a picker used to select and remove them for cultivation. Clearly, one major advantage of this single cell/bead selection system is that it obviates the need to cultivate and assay all candidates using laborious large-scale screening methods.

The majority of the novel screening methods introduced at the symposium require some form of molecular tagging. However, analytical methods such as are used in earth remote sensing systems cannot enjoy such a luxury, and it transpires that infrared spectrometry in combination with the implementation of modern chemometric methods for multivariate analysis (DRASTIC – Diffuse Reflectance Absorbance Spectroscopy Taking In Chemometrics), which exploits natural spectral characteristics of molecules of interest for their identification, is sufficient to discriminate and quantify target determinands in complex fermentation broths *in situ* without separation or purification (Douglas B. Kell; University of Wales, Aberystwyth, UK). In a model fermentation system the carbocyclic nucleosides aristeromycin and neplanocin A, produced by *Streptomyces citricolor*, could be distinguished rapidly and quantitatively with these methods during a titre improvement programme.

One view had it that if today was the era of the multiwell plate, 'tomorrow' was that of molecular screening while the future would involve database mining and forced molecular evolution. Overall, the conclusion is that HTS methods will continue to be of great importance in the development of clean and efficient microbial biocatalysts, and that robotics, bioinformatics and data processing will be significant drivers in speeding the progress of biotransformations as agents of chemical change.

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Biotechnology patents and morality

R. Stephen Crespi

The question of whether considerations of morality should influence the patenting of inventions in biotechnology is a topical one for those involved in patenting and patent law in Europe. It is also of interest to scientists, business people and even politicians. For patent attorneys, whose task it is to present the case for the originality and patent-worthiness of the work of inventors, moral debates are a distraction from their primary mission. Disputes of this kind are highly subjective and difficult to resolve quickly. The resulting sense of frustration can be seen in the following personal viewpoint of one patent attorney.

Patent law is a subject that usually arouses little excitement. Patents are important for those in the business of research and technological innovation but the procedures for obtaining them are seen as complex, slow and rather dull to anyone but patent attorneys and lawyers. Biotechnology is changing this perspective. As the biotechnology companies contend with one another in the courts, an intriguing case law is emerging. Add to this the vociferous opposition from special interest groups to patents on living organisms and genes, and the subject begins noticeably to warm up. Biotechnology has certainly brought the patent law to life.

The moral dimension in patent law

Patent law exists to stimulate the advancement of technology. It is grounded in the assumption that new processes and products that provide material benefits and enhance the quality of human life in other ways are desirable and should be encouraged. But other ideas must also be given due weight if science and technology are not to lead to a sterile culture of consumerism. One of these is the ethical dimension of new technology, a topic that has captured the attention of many persons, public officials and committees, as well as protest groups.

Patent law is not silent on the question of morality. Morality was brought into the patent law at least from the time of the Strasbourg Convention (1963) in terms that were later enshrined in European law through the European Patent Convention (EPC, 1973). The EPC has now largely eclipsed the existing parallel national patent systems in Europe and is the one most used by those who invest heavily in science and technology, especially biotechnology. Article 53(a) of the EPC excludes from patentability any invention 'the publication or exploitation of which is contrary to morality or ordre public'. The corresponding national patent laws contain essentially the same provision.

Controversy is the daily fare of patent law. Procedures for objecting to the grant of specific patents allow ample scope for virtually anyone but the frivolous objector to have his day in court. EPC Article 53(a), in particular, has provided a convenient international forum in which the Greens, animal rights campaigners and others have not been slow to promote their particular philosophies. The Opposition to the Harvard oncomouse European patent is the best-known instance of this, there being no fewer than 17 Opponents, among which the British Union for the Abolition of Vivisection, and Compassion in World Farming are the two most prominent.

Most of the arguments in patent disputes can be resolved by factual enquiry and legal logic. Moral arguments are not so easily dealt with. Many commentators dispute the relevance of morality to patent law. They also question which set of moral principles is applicable in this context, given the diversity of ethical theories that vie for favour among moral philosophers today.

The morality of patenting

In considering morality in a patent context, it is necessary to avoid a common confusion. EPC Article 53(a) is concerned with the morality of certain specified actions. Leaving aside the publication aspect, it is the act of actual exploitation of the particular invention to which the moral test must be applied. Whatever the ultimate intentions of the patent holder, the morality of the act of patenting does not come into

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PII: S0167-7799(97)01028-7

the question. The belief that it is wrong to patent certain substances, organisms or processes calls for an ethical judgement that is outside the patent law itself and therefore one that patent officials or judges cannot be called upon to make.

Patenting an invention does not commit the patent holder to any particular course of action. Having a patent may be an encouragement to exploit an invention but exploitation can take place even without a patent, assuming other laws and regulations permit. But if one were to assert that biotechnology patenting is a morally dubious activity in itself, the context in which it arises must be considered. First, there is the research, which must be successful or there will be no invention to patent. Then comes the patenting step and after that the publicity and commercial exploitation. Unless we believe that it is wrong to do this kind of research, or that it is wrong to publicize and exploit the results of this research, or that both of these are wrong, it is a strange kind of ethical selectivity to argue against patenting in isolation.

Patenting, as such, is neither wrong nor right, but could be classed as 'ethically neutral'. To refuse a patent would be a futile gesture that would not by itself stop the invention being put to practical use. However, if society were to judge that the practice of a particular invention deserved to be banned by law then nobody would bother to patent it.

The various ways in which objections to biotechnology patents are being formulated are summarized below.

'Patenting life' – the power of the slogan

There is a basic objection to the idea of 'patenting life' and thereby 'owning life'. Science cannot tell us precisely what life is but we ordinarily use the word as an abstraction from concrete living things. It follows that 'patenting life' is a meaningless notion since the law does not allow the patenting of abstractions. This unfortunately does not prevent 'patenting life' being used as an effective slogan to delude people into thinking that a sinister move is being made to monopolize the very stuff of life. This tactic has powerful emotional force. If 'life' can be patented and owned, the objectors say, it is being treated as a 'commodity'.

Were the objection to be framed in a more honest way as one against the patenting of living things it would immediately lose most of its force, as living things can indeed be commodities, such as yeast for bakers and brewers and edible plants and animals for farmers. Making this statement in no way commits one to a 'reductionist' philosophy that views living things as mere objects.

When confronted with the fact that one does not patent 'life' but only living organisms (modified in some way from what Nature produces) the objectors tend to shift their ground somewhat. Realizing, of course, that farmers actually do own the plants they have grown and the animals they have bred and reared, the objectors draw a distinction between owning specific plants and animals and the ownership of 'whole species' that they claim is achieved by patenting. This is a clever debating point but it has no substance. A species is a biological classification and not an entity in its own right. The same, incidentally, can be said of the plant 'variety'. These terms are also abstractions; nobody ever meets a species or a variety but only individual members of these groups. Furthermore, biological classification systems have not yet accorded species status to transgenic plants and animals.

Patenting microorganisms, plants and animals

Microorganisms

Those who object to the patenting of living matter will not allow any exception to their rule. The objection to patenting microorganisms is usually merged into the general charge that patenting 'life' implies a failure to respect life. But society cannot have bread, wine, antibiotics and vaccines without the industrial use of microorganisms. These products are beneficial and to produce them more effectively by means of improved and patentable strains of microorganism is also meritorious. Patenting the organisms harms nobody.

Plants

Nor does the morality argument sit comfortably when applied to the patenting of plants. Usually, the morality objection slides into one of challenging the use of genetically manipulated plants on grounds of public safety. Important though the question of safety undoubtedly is, it is primarily a matter of complying with other laws and regulatory procedures that have been established as matters of public policy. It would doubtless be immoral to act irresponsibly in matters affecting public health but the legal control of this has nothing to do with patents.

Animals

The morality argument appears more pertinent when it comes to the patenting of animals. Here we are dealing with a sentient being and we have to ask whether that makes a difference to the moral issue. As one American journalist put it, when in April 1988 a US patent was granted on the Harvard oncomouse, 'Last month the Government granted its first patent on something that can look you in the eye'. It is necessary first to address the metaphysical objection that an animal should never be reduced to the status of an 'invention' let alone a patentable one.

The term 'invention' in patent law has to serve for a wide variety of items that are presented as new and improved over what is already known and used, i.e. over the 'prior art'. Some inventions are startlingly new in their own field but most are improvements or modifications of existing processes and products. These latter kinds of invention may be less original than the 'pioneering' inventions but all that concerns the patent law is whether their difference from the prior art required inventiveness to accomplish or was 'obvious' to the skilled person.

In the case of a transgenic animal it is this difference from the prior art upon which the legal examination

focuses. Since this difference does not occur naturally it is reasonable to describe it as a work of human ingenuity, as human handiwork rather than that of Nature. The US Supreme Court made this point in 1980 in approving the granting of the first patent on a genetically manipulated strain of bacterium (*Chakrabarty pseudomonas*) and it is equally applicable to higher life forms. The inventor certainly does not undervalue the work of God or Nature, which provided the starting point, nor do they claim to have 'created' the whole animal. Of course, the patent has to cover the modified animal as a whole since the difference cannot be used in isolation.

Animal transgenics involves the genetic modification of two main types of animal, laboratory test animals and farm animals. Each of these requires its own special treatment from the moral point of view.

Laboratory animals

The Harvard oncomouse is genetically programmed to be more sensitive to carcinogens. In this respect the oncomouse is superior to previously used types of laboratory mouse, which may mean that scientists can use fewer mice than before. It is nevertheless an emotive case, which reinforces the dilemma over the general question of sacrificing animals in the quest for cures for human diseases.

The moral argument against animal experimentation is that the animals are used as mere tools for human ends and in ways that involve suffering. The animal's pain and in some experiments the indignity for the animal are distressing to many people. However, if this is a real moral issue for humans and not just one of sentimentality, it has nothing to do with the question of patents for improved test animals. It is the fundamental issue that society must settle rather than the side issue of patenting.

Patenting is under attack because the system presents an easy target and gives the objectors a platform for much publicity. But the patent officials have to decide the question against a background in which the practice of breeding and using laboratory test animals is widely accepted as permissible and indeed necessary if medical science is to advance. If that practice is not immoral *per se* the objectors have the burden of convincing the authorities that it becomes immoral by the introduction of genetic manipulation or some other technique of biotechnology in the breeding process.

Farm animals

Respect for and appreciation of animals, and concern over the conditions under which animals are intensively reared and transported, are shared by both vegetarians and non-vegetarians. These concerns charge the emotional atmosphere but have little or nothing to do with the subject of transgenic animals and patenting.

In Britain, ethical issues involved in various aspects of this technology have been addressed by two public committees.

The first of these, the Polkinghorne Committee[1], was asked to consider 'the moral and ethical concerns (other than those related to food safety) that may arise from the use of food products derived from production programmes involving such (transgenic) organisms'. The Committee did not address wider ethical matters but did discuss the idea of 'moral taint' attaching to foods produced with the aid of genetic modification and deriving from the 'unnaturalness' and any animal suffering that might be involved.

In considering the element of unnaturalness the Committee identified, as the key factor, 'the status of the human genetic material inserted into the farm animal'. The committee distinguished beween human genes as existing in the human body and 'copy genes of human origin'. The procedure for cloning the gene results in an enormous dilution effect (10^{55}) so that the inserted material is overwhelmingly in the form of synthetic copies of the original. If mRNA cloning is used, literally nothing remains of the original gene or message. Accordingly, the essential phenotype of the recipient species is not altered. On the basis of these essentially scientific facts, the Polkinghorne Committee rejected the idea of 'a moral taint that would warrant a total prohibition on genetically modified food use'.

The second committee, the Banner Committee[2], considered the ethics of novel farm animal breeding techniques. The committee asked whether there are 'intrinsic' objections to certain types of genetic modification that cannot be overcome by 'consequentialist' arguments. According to the consequentialist view of ethics, the morality of an action is to be judged (only) by its consequences. This usually entails balancing the benefit against the harm resulting from the action. An intrinsic objection is one not related solely to the consequences of a particular practice or action but to the practice or action itself.

In genetic modification, the Banner Committee considered that there is a line between what is acceptable and what is not. While this cannot be drawn sharply, the committee felt that 'harms of a certain degree or kind ought under no circumstances to be inflicted on an animal'. One prominent theme in this connection is that of naturalness, contrasted with modifications that threaten the achievement of the animal's 'natural ends or good' or do not respect its 'essential nature and well-being' or its 'natural characteristics and form'.

For example, the use of gene technology to increase the protein content of cow's milk or to cause poultry breeding stock to produce only female chicks could be achieved while still respecting the essential nature and well-being of the animal. These were therefore not considered intrinsically objectionable modifications. However, to increase the efficiency of food conversion in pigs by reducing the sentience and activity of the animal was to disregard the ends and purposes that are natural to pigs and was intrinsically unacceptable.

Asking whether there might be reasons for prohibiting patents in this field, the Banner Committee rejected most of the anti-patent arguments. However, having concluded that some instances of genetic modification are acceptable and some not so, it felt that the

patent law should discriminate between these. Having passed this heavy burden to the patent authorities the Banner Committee went on to dismiss the provision in the proposed EU Directive[3] on this subject (see later), which would have denied patents for 'processes for modifying the genetic identity of animals which are likely to cause them suffering or physical handicaps without any substantial benefit to man or animals, and animals resulting from such processes'. The committee were not convinced that this was a workable and appropriate provision since it left so much open to question.

This Directive was voted down by the European Parliament in March 1995 but a revised version was re-presented by the Commission in December 1995. In Article 9-2(b) of the new draft the above provision has been retained, but with the added wording: 'insofar as the suffering or physical handicaps inflicted on the animals concerned are out of proportion to the objective pursued'.

Patenting material derived from human tissue

Patents have been granted on materials isolated from human tissues and some have been challenged, including the following two examples.

The John Moore case

John Moore has expressed concern over the fact that cells taken from his spleen have been immortalized and patented by his physician and the University of California. John Moore's cancerous spleen had been removed by his physician who then, apparently without his patient's consent, researched its cell content and developed a useful and patentable cell line. The patent (US Patent No. 4 438 032) covers a single cell suspension of the Mo cell line and various proteins produced from it.

John Moore sued both the physician and the University. On the intellectual property ownership issue Moore's claim was not upheld by the California Supreme Court. The court decided that he was not entitled to claim ownership of the intellectual property in the cell line. The patented cell line, though developed from the spleen cells, was held to be different from the cells actually removed from the patient. Moreover, the court was of the view that to grant to the 'donor' of an organ the intellectual property in anything developed from it would inhibit research of this kind. John Moore nevertheless continues to believe that others have patented and now 'own his genetic essence'.

The relaxin case

The Green Party opposed European Patent No. 112 149 granted to the Howard Florey Institute of Experimental Physiology and Medicine for a gene sequence coding for human relaxin[4], a hormone involved in reproduction. The gene was isolated from ovarian tissue removed in the treatment of an ectopic pregnancy.

One of the Opposers' arguments was that it is 'an offence against morals to exploit the pregnancy condition of a woman by removing tissue from her ovary and using it as the basis of a profit-oriented technical process'. The Opposers also raised the bizarre (in my opinion) objection that the use of the ovarian tissue for the purposes of the patent 'involves dismemberment of women and their piecemeal sale to commercial enterprises'.

The Opposition Division roundly rejected these objections. The tissue had been provided with patient consent; it was perfectly acceptable to use such material; DNA is not 'life'; a human being cannot be reconstructed from the sum total of human genes; and many other reasons were given for dismissing the arguments as thoroughly misplaced.

Are there genuine reasons for concern?

It is hard to see what real harm or injury to the original donor of the tissue is caused by the patenting of a cell line or a gene derived from tissue in these circumstances or, indeed, what reason exists for any repugnance to this sort of research and development. And yet, the arguments in the John Moore case were apparently prominent in the last-minute struggle to survive of the proposed European Council Directive on the legal protection of biotechnological inventions. With the John Moore case in mind, the Conciliation Committee, set up jointly by the European Council and Parliament, inserted various Recitals into the final text of the Directive[3], for example Recital 12 reading as follows:

> Whereas, in the light of the general principle that the ownership of human beings is excluded, the human body or parts of the human body as such, for example a gene, protein or cell in the natural state in the human body, including germ cells and products resulting directly from conception, must be excluded from patentability; whereas, however, an invention incorporating industrially applicable parts obtained in a technical manner from the human body in such a way that they can no longer be ascribed to a particular individual may not be unpatentable because of the human origin of such parts, even where the structure of these elements is identical to a part of the human body, it being understood that parts of the human body from which such parts are derived are excluded from patentability.

This wordy statement was the result of last-minute intensive effort to produce a text that would satisfy the various factions in the European Parliament and save the Directive. The ethical reasoning on which Recital 12 was based is strange indeed. First, what possible connection could there be between owning human beings, e.g. slaves, and the patenting of a DNA sequence? Second, the practical circumstances in which anyone could conceive of patenting, or would want to patent, anything *in situ* in someone's body are (to say the least) difficult to imagine. Third, how anything isolated from a particular individual (e.g. John Moore), and replicated, sub-cultured and adapted for

use in an industrial process, could possibly remain linked in some way to that individual stretches the imagination to breaking point.

Ethical views of various organizations

Ethics in science and medicine

The consequentialist view of morality seems to be the 'establishment' view of science and medicine. The use of experimental animals is justified in the following exemplary quotation[5] by Lord Adrian, President of the Research Defence Society: 'The use of living animals in scientific research can be considered justified if it is likely to produce appreciable benefit to society, if there is no other way to conduct the research in question and if all reasonable steps are taken to keep any distress or suffering to a minimum'.

A very large percentage of the public would probably agree with this position. It also appeals to lawyers. In the European patent application for the Harvard oncomouse the Technical Appeal Board instructed the Examining Division of the European Patent Office (EPO) to consider the morality issue under Article 53(a). The Board suggested that the decision 'would seem to depend mainly on a careful weighing up of the suffering of animals and possible risks to the environment on the one hand and the invention's usefulness to mankind on the other'. The Examining Division decided that the likely benefit to cancer research outweighed the other factors and granted the patent.

This 'balancing principle' is criticized by the Opponents of this patent who say that an intrinsically immoral act cannot be justified by the fact that some good consequences ensue. This argument rests on the unproven first premise that it is immoral to alter the genes of animals and especially so if it results in animal suffering.

Official and patent professional views

The European Patent Institute (EPI) has stated[6] the professional view on the subject from a practical standpoint. The EPI support the official view of Article 53(a) as expressed in the EPO Guidelines, where it is stated that 'This provision is likely to be invoked only in rare and extreme cases. A fair test to apply is to consider whether it is probable that the public in general would regard the invention as so abhorrent that the grant of patent rights would be inconceivable.'

The EPI also endorse the view of the EPO Opposition Division[7] that 'Only in those very limited cases in which there is an overwhelming consensus that the exploitation of an invention would be immoral may an invention be excluded from patentability under Article 53(a).'

As to the test suggested by the Appeal Board, which is evidently to be used as a standard for transgenic animal cases, the EPI observes that it will often be very difficult if not impossible for patent officials to assess the benefit against the harm of particular inventions, since these may be fully revealed only in the longer term and not at the early stage at which patent applications are examined. The EPI much prefers the 'overwhelming consensus' test as the ultimate criterion for the purposes of Article 53(a) and sees the balancing principle as just one factor contributing to the assessment.

The Nuffield Council

The Nuffield Council on Bioethics has addressed patent issues in Chapter 11 of its recent report[8]. This is a lucid summary of the issues involved in patenting inventions derived from human tissue and it discusses the following options for resolving problems.

One option is to remove the morality issue from the remit of the Patent Office and to leave it to national courts. The Council notes the disadvantages of this option, especially the likelihood of different national treatment and the resulting confusion. It seems to me that the legal mechanism for this option is far from clear for European patents, i.e. those granted under the European Patent Convention (EPC). National courts do not become competent to deal with EPC patents until these have left the jurisdiction of the EPO. It would be a most strange provision in the EPC itself that could not be raised by the EPO either during normal examination of the patent application or in Opposition proceedings after the patent has been granted.

Another option is the 'light approach', favoured by two former Comptrollers of the UK Patent Office. This is a variant of the first option in which the Patent Office invoke the objection only in extreme cases, leaving it to national courts to sort out remaining problems. This corresponds to the present attitude of the EPO in examining patent applications. How it would work in the face of a determined third-party Opposition to the patent is far from clear.

A third option is to transfer the responsibility from the patent examiners to a specially constituted ethics committee, which would become part of the official process either before or after grant of the patent.

The fourth option, which is most favoured by the Nuffield Council, is the creation of a protocol to the EPC setting criteria for national courts in applying the immorality exclusion. This might avoid the problem of differing national treatment but I think it is open to the same difficulty as raised against the first option. Bearing in mind the predominant role of the EPC in the European patent scene, any scheme that retains the immorality exclusion in the law of the EPC but aims somehow to exclude or mitigate its rigorous application during the European phase of the patenting process may not be viable.

The Council of Europe Bioethics Convention

The Council of Europe has prepared a 'Draft Convention for the Protection of Human Rights and Dignity of the Human Being with regard to the Application of Biology and Medicine: Bioethics Convention'[9]. From its very title and general character, this Convention is concerned with matters affecting the human being at the personal level. Provisions relating to the disposal of human tissues, organs and other body parts are therefore subsidiary to its main aims. The Convention is at present in

draft form (Document 7124, as amended by Opinion No. 184 of the Parliamentary Assembly). Article 11 of the Convention (Prohibition of financial gain) states that 'The human body and its parts shall not, as such, give rise to financial gain'. The Explanatory Report (Document 7214, page 24) makes clear that this Article is particularly concerned with organs and tissues removed from the human body and the sale of these for 'financial gain to the person from whom they have been removed or for a third party, whether an individual or a corporate entity such as a hospital'.

In a draft Opinion of the Reporting Committee (Committee on Science and Technology Document 7210), Article 11 is considered as extending to the patenting of the human body or its parts (at Section D21, page 12, where comment is also made that touches upon the distinction between discovery and invention in patent law).

The extension of Article 11 into the sphere of patent law goes beyond the proper remit of the Convention and is unwarranted. Patent law issues that arise in the field of industrial processes and products do not conflict with the protection of human rights and dignity. The opinion expressed concerning discovery and invention is oversimplified and contrary to both the official and private view prevailing among those involved in patenting and patent law.

According to paragraph 18 of the Explanatory Report, the scope of the Convention is restricted to human medicine and biology and excludes plant and animal biology 'insofar as they do not concern human medicine and biology'.

In accordance with its objectives the Convention rightly addresses medical intervention procedures carried out on the human being. It should not venture into industrial biotechnology.

Other objections: patenting genes

Those who object to biotechnology patents do not restrict themselves to raising moral objections. They are also ready to deploy patent law arguments as part of their strategy. This can be seen in their criticism of gene patents.

Discovery or invention?

The law says that inventions are patentable but discoveries are not. The objectors argue that because genes exist in Nature they cannot be invented but only discovered. The distinction between discovery and invention is difficult to define in any of the sciences of Nature because the act of discovery so closely underpins the resultant practical application that constitutes the invention. As ethicist W. Ch. Zimmerli has put it[10], 'every scientific discovery, if made technologically applicable, becomes an invention'.

The official patent authorities are not prepared to dismiss the isolation of genes as mere discovery. There are many judicial statements to this effect (which would require more space to expound) but the objection persists.

The question of novelty

Another argument against patenting genes is that, because of its pre-existence, a gene cannot fulfil the patent law test for novelty. But this test is framed in terms of availability to the public. Thus it focuses only upon what is already in the public domain through public disclosure or use before the filing of the patent application.

Genes do not easily fit into this scheme. To be made available to the public the gene must first be isolated, preferably sequenced and cloned. The contribution to the art on which gene patents are based is the making of the gene available in a form that can be used to produce an expression product, and to produce this in quantity, for example, as a commercial pharmaceutical product. Alternatively, the cloned gene can be used to transform an organism of another species, giving rise to new products, e.g. transgenic plants and animals. Genes are therefore a special case of the broad class of naturally occurring substances that in appropriate circumstances can be patented. Mere pre-existence of the substance, in association with vast quantities of other materials, is insufficient to overcome the objection. This is the official legal view.

The question of inventive step

Those who describe DNA sequences as 'mere discovery' are now latching on to the argument that however difficult gene cloning was in the early 1980s it is becoming routine in the laboratories of today. Whatever the state of current general skill and knowledge in relation to these techniques, this argument is only relevant to the question of inventive step, i.e. to how much ingenuity is required to isolate genes. This evaluation must always be made case by case and no broad general dismissal is justified.

Do we actually patent genes?

In answering this question patent officials have recognized the distinction between the genes of lower organisms and those of higher life forms. They have noted that, whereas prokaryotic genes are transcribed in full, the messenger in eukaryotic gene transcription is an edited form of the primary RNA transcript. Consequently, the DNA made by enzymatically copying mRNA into cDNA is not the natural gene as such.

In summary, objections of the type considered here have so far been singularly unsuccessful when applied against European patents. Nevertheless these interventions add to the expense of patenting and cause uncertainty to the innovators.

Conclusions

It is undeniable that biological research throws up profound moral questions, especially in the field of human reproductive technology. However, the relevant debates should begin at the right point, with the research and its potential uses, and not with the wholly subordinate question of patents. The patent issue is no short cut to the resolution of the more difficult questions, and until these have been properly

addressed, patenting should not be singled out for attack.

Removal of the current morality provisions from the patent law is not a practical possibility in the foreseeable future. It is unfortunate that the legislators have put these provisions in such an inappropriate place but those responsible for administering the law should interpret the exclusions narrowly and not allow them to be used for disruptive purposes.

Judging from recent experience, attempts to put specific provisions into the law as to what particular types of invention are to be unpatentable are likely to be the subject of interminable controversy. At present, the debate is provoked to suit the agenda of the Greens, animal rights campaigners and their supporters. Once started there is no reason why it should not extend into other areas of moral dispute. For example, the question of patenting abortifacient drugs raises for many people far more important moral questions than those of animal welfare. Pandora's box is better kept shut.

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introductory overview

The marine biosphere: a global resource for biotechnology

Don A. Cowan

The marine biosphere is often considered as one of the richest of the earth's innumerable habitats, yet is one of the least well-characterized. Because of its diversity and scale, it offers enormous current and future opportunities for non-destructive exploitation within the many facets of modern biotechnology.

Biodiversity

Biotechnology is a 'natural product' of biodiversity! New products and processes, the keys to the future development of biotechnology, are as dependent on new sources of biomaterials as on the scientific ingenuity required to discover, evaluate and develop such resources. It must follow therefore that the greater the biodiversity, the greater the opportunity for discoveries that may ultimately be translated into valuable biotechnologies.

Marine biodiversity is extremely high, a direct consequence of the extraordinary variability of the marine biosphere. This biosphere encompasses a huge thermal range (–1.5°C in Antarctic waters to temperatures in excess of 100°C in shallow and 350°C in deep hydrothermal systems), pressures ranging from 1 to over 1000 atmospheres, nutrient variations ranging from eutrophic to oligotrophic, and extensive photic and non-photic zones. This diversity has facilitated extensive speciation at all phylogenetic levels, from microorganisms to mammals! With intensive research programmes in many areas of marine macrobiotic diversity, discoveries of new macrobiotic species are relatively frequent. For example, the discovery of unique deep submarine hydrothermal vents has led to the identification of a catalogue of novel species, including mussels, clams, shrimps, polychaetes, vestimentiferan tubeworms, anemones and crabs[1]. Microbial diversity studies, which for reasons of technical complexity usually lag far behind macroorganism research, have also shown unique speciation in these sites, including hyperthermophilic archaea and shrimp epibiotic bacteria. While marine hydrothermal vents are highly specialized biotopes, which make only a small contribution to global marine biomass, the importance of such sites to the field of biotechnology should be measured by genetic diversity rather than population volume.

It is clear that the microbial diversity of many well-known and high volume marine biotopes is still poorly

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PII: S0167-7799(97)01027-5

understood. Recent studies of microbial diversity using direct analysis of rRNA sequences have shown that marine coastal, surface and subsurface waters contain very large populations of microbial picloplankton[2], and that this biomass fraction contains a high abundance of archaeal species, virtually none of which have ever been isolated by classical microbiological methods[3]. The presence of archaeal species as a significant proportion (up to 34%; Ref. 3) of total prokaryotic biomass in the vast volumes of the cold Antarctic oceans indicates that one of the most abundant components of the earth's microbial biota has hitherto escaped notice.

The lessons from these discoveries are inescapable. The world's oceans do indeed represent a biodiversity resource of huge dimensions but about which we know relatively little. For example, it is estimated that over 95% of the all extant fish species are known, but less than 5% of bacterial species[3]. The significance of this resource in terms of global gas exchange (methanogenesis, CO_2 fixation or evolution, O_2 evolution), nutrient and elemental cycling, biomass and food production (aquaculture and fisheries), marine products (polymers, organics, bioactive compounds, biocatalysts) and economic processes (bioremediation, biomining and desulfurization) is widely perceived but often largely unresearched.

Areas of biotechnological development

The potential of marine organisms for commercial development and exploitation impinges on virtually every area of biotechnology. The following examples are presented merely as a flavour of the diversity of opportunities that are current topics for research and development.

Biomedical products

The biological diversity of the marine biosphere offers enormous scope for the discovery of novel natural products, many of which are potential targets for biomedical development. Commercial interest in the development of new pharmaceutical and pharmacological agents has ensured that this is the most actively developing area of marine biotechnology, this view being supported by a survey of the literature over the past five years, which indicates hundreds of papers on the identification, structural elucidation and bioactivities of marine natural products. The marine sponges and macroalgae have been particularly productive sources of bioactive natural products, including for example, antitumour agents (theopederins, cribostatins and halogenated monoterpenes), immunosuppressives (microcolins and discodermalide) and anti-infectives (the antimalarial, axisonitrile). Certain products, such as didemnin B, a cyclic depsipeptide from the Caribbean ascidian *Trididemnum solidum*, have advanced to clinical trials[4]. The development of commercially supported high-throughput screening programmes, based on enzyme inhibition and receptor agonist/antagonist assay systems, has led to the discovery of novel topoisomerase, protein kinase, phospholipase, protease and protein phosphatase inhibitors, with considerable potential for therapeutic application as antineoplastics, antivirals and anti-inflammatory agents[5].

Antifreeze proteins

The discovery that the Arctic and Antarctic fish generate high concentrations of 'antifreeze' proteins that play an important role in thermal hysteresis and the prevention of ice formation was a startling example of environmental adaptation. These proteins, which have now been found in many cold-adapted organisms, offer significant potential for biotechnological exploitation. While the exogenous addition of antifreeze proteins in the manufacture of frozen foodstuffs to enhance freeze–thaw properties has been considered, the *in vivo* expression of antifreeze protein genes in transgenic plants or animals offers wider opportunities for expanding crop-production or food storage properties[6]. The generation of transgenic Atlantic salmon expressing the type 1 AFP (antifreeze protein) gene from flounder is a significant step in this development pathway.

The functionally related ice-nucleation proteins derived from psychrotrophic bacteria[7] have already been commercially employed in the process of snow-making. These proteins also have potential applications in the production of ice-cream and similar food stuffs, in cloud-seeding (as a replacement for AgI) and, via transgenic expression, in the prevention of frost damage in economic crops.

Bioadhesives

Many marine invertebrates (e.g. mussels and barnacles) secrete adhesive proteins to facilitate attachment to solid substrates. The sequencing of these proteins identified an unusual decapeptide repeat sequence [(Ala-Lys-Pro-Ser-Tyr-Hyp-Hyp-Thr-Dopa-Lys)$_{75}$]. Chemically synthesized analogues of this natural sequence are at present being developed for a variety of biotechnological and biomedical applications, including tissue bonding and wound repair preparations[8]. These synthetic peptides also offer a novel method for screening compounds for antifouling properties.

Accessing biodiversity

Traditional methods for accessing biodiversity, whether for the investigation of phylogeny, physiology or biochemistry, involve collection and *ex situ* analysis. Until recently, studies of microbial diversity typically required the preparation of pure cultures, using solid or liquid media. At this point, modern molecular methods such as DNA–DNA hybridization, rRNA gene sequencing or protein electrophoretic analysis could be used to establish taxonomy, much as would be applied to a sample of marine macroflora or fauna. Similarly, the generation of pure microbial cultures typically precedes any biotechnological investigation, whether screening for specific biocatalytic activities or for therapeutically active compounds.

At least in the microbial sector, this requirement for species separation has been a major impediment to the comprehensive analysis of biotechnological potential. The current belief that less than 10% (and possibly less than 0.1% in certain environments) of the microbial diversity in any environmental sample is culturable[9] is evidence of the gulf between the potential of the resource and our present ability to access it.

Recent developments in molecular technology offer a route to the analysis of natural microbial biodiversity which circumvents the limitations imposed by the need to isolate and culture individual species. The efficient extraction of total DNA from an environmental sample provides a mechanism for the simultaneous analysis of multiple genomes, potentially representing the total microbial diversity within that sample. Homologues of known gene sequences can be identified and isolated by hybridization probes or PCR amplification, while gene products of any type may be identified by screening of expression libraries. The latter approach, currently in its infancy, is highly adaptable and, depending on the ingenuity of the screening protocol, might be used for identifying novel enzymes, bioactive peptides or even the products of linked multi-gene complexes.

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Progress in understanding the fish pathogen *Aeromonas salmonicida*

Brian Austin

Aeromonas salmonicida is the causal agent of furunculosis in various fish species. Detection methods include culturing, serology and molecular biology techniques. Controversy surrounds its possible independent existence in water; enzyme-linked immunosorbent assay and the polymerase chain reaction have detected *A. salmonicida* in the absence of colony-forming units, but cells that are non-culturable may be significant to fish pathology. Furunculosis is probably transmitted by the pathogen's entry into gills, mouth, anus and/or surface injury of fish through contact with infected fish or contaminated water. Disease-control is possible by good husbandry practices, disease-resistant stock, improved diets, nonspecific immunostimulants, antimicrobial compounds and vaccines.

A diverse range of bacteria, fungi, protozoa and viruses is associated with diseases of marine animals. Among the bacteria, *Vibrio* spp., including *V. anguillarum*, *V.* (*Photobacterium*) *damsela*, *V. ordalii* and *V. salmonicida*, are commonly associated with a haemorrhagic septicaemia, termed vibriosis[1]. The fungal species *Ichthyophonus hoferi* affects many marine species, causing a disease known as 'ich'[2]. One of the most serious virus diseases, affecting many fish species in the sea, is lymphocystis[2]. All these pathogens cause losses in wild and farmed fish stocks. In terms of the application of biotechnology to the control of fish disease, emphasis has been placed on one bacterial fish pathogen, namely *Aeromonas salmonicida* (Box 1).

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PII: S0167-7799(97)01026-3

Box 1. Characteristics of *Aeromonas salmonicida*[1]

Gram-negative, non-motile, fermentative rods, which typically produce brown diffusible pigment on protein-containing medium such as brain heart infusion agar (BHIA). Incapable of growth at 37°C. On BHIA it may develop any of three different colony types, termed 'rough', 'smooth' and 'G-phase' because of the presence or absence of an external proteinaceous layer (A-layer). Cell-wall-defective/deficient forms (L-forms) have been recognized. Atypical isolates may be slow in pigmenting and nutritionally fastidious, requiring blood-products for growth. By definition, cells do not occur in surface water, and are pathogenic to fish.

A. salmonicida is the causal agent of furunculosis, and is one of the oldest known fish pathogens[1,3]. The pathogen has a worldwide distribution, causing infections in representatives of many families of fish, including Anoplopomidae, Cyprinidae, Salmonidae and Serranidae. Classical furunculosis involves a haemorrhagic septicaemia, including the presence of furuncles (boils) on the flanks. The seriousness of furunculosis to aquaculture (the rearing of aquatic species in controlled conditions) is illustrated by the epidemic in 1991–1992, which led to the loss of ~10 000 tonnes (roughly equivalent to 25% of the total production) of Atlantic salmon (*Salmo salar*) in Scotland. Although troublesome initially in freshwater, *A. salmonicida* has emerged as a major pathogen of salmonids and other fish, such as dabs and flounder, in the sea[4,5]. In these fish, the disease may be of a different form from classical furunculosis, as it results in ulcers, and the aetiological agent is deemed to be 'atypical'[4,5]. Three outstanding problems in *A. salmonicida* biology remain to be resolved: the determination of the precise location and form of the organism in asymptomatic/carrier fish, the location of the reservoir of the pathogen in the aquatic environment, and the availability of effective disease control measures, especially for fish other than salmonids.

Detection

A. salmonicida is difficult to recover from fish that do not show clinical signs of disease, and from the aquatic environment, even during the epizootics of furunculosis. Routinely, cultures may be grown on tryptone soya agar or brain heart infusion agar following incubation at 15–25°C. Atypical isolates often need the inclusion of blood or serum in the isolation medium[1]. Pre-incubation of pathological material for 24–48 h in tryptone soya broth[6] followed by the use of Coomassie Brilliant agar improves recovery of the pathogen[6,7]. However, an effective selective medium remains to be described. Alternative approaches to culturing for the detection of *A. salmonicida* and diagnosis of furunculosis have included serology and molecular biology techniques.

Serology has been used successfully on pure and mixed cultures and with pathological material to confirm the presence of *A. salmonicida*. Methods range from slide agglutination[8] to the latex agglutination test[9] and enzyme-linked immunosorbent assay (ELISA)[10]. Latex agglutination, of which one system has been commercialized, may result in diagnoses within 2 min to 2 h for pure/mixed cultures or heavily infected fish tissues, respectively. ELISA enables reliable diagnoses in 30–60 min, and appears to be effective for use with asymptomatic carrier fish[1,10]. The ongoing debate concerning the relative merits of monoclonal and polyclonal antibodies in serological techniques remains to be resolved.

DNA probes have the ability to detect *A. salmonicida* in clinical and environmental samples. Moreover, a DNA fragment specific to *A. salmonicida* was incorporated into a polymerase chain reaction (PCR) technique and used to detect approximately two cells[11]. By means of PCR and a specific DNA probe, the presence of *A. salmonicida* has been reported in effluent, water, faeces and sediment from a commercial freshwater Atlantic salmon farm in Ireland[12,13]. In contrast, culturing only recovered colony-forming units from clinically diseased fish. PCR technology has enabled the demonstration of DNA, four months after the use of furunculosis vaccines, in the kidney and spleen of Atlantic salmon (*S. salar*)[14]. Both serology and molecular methods suffer from the disadvantage that they may not distinguish living (pathogenic) from dead cells, such as those incorporated in many vaccines. Therefore, in the long term, the increasing sensitivity of modern detection methods may not be too helpful with the management of disease caused by *A. salmonicida*.

The possible survival of *A. salmonicida* in water has been extensively researched[1]. The data suggest that the pathogen can survive through varying periods and temperatures in fresh, brackish and seawater, and the underlying sediment. A consensus view is that there is a concomitant reduction in pathogenicity when the organism leaves the fish and enters the aquatic environment[15]. Sakai has suggested that the long-term survival of *A. salmonicida* in the aquatic environment may reflect electrostatic charge differences on individual cells, with positive and negative charges on avirulent and virulent cells, respectively[15]. Virulent cells may be able to survive under starvation conditions in river sediments. A decline in numbers might be caused by the spontaneous occurrence of avirulent free-living cells, which enter a dormant phase due to a lack of nutrients[15]. Furthermore, these free-living cells could represent a transitional form, leading eventually to a loss of viability. Interestingly, the onset of the dormant/non-culturable phase may be delayed by the presence of the amino acids arginine and methionine[16]. Although the possible presence of dormant/non-culturable cells in the aquatic environment has been vigorously debated, there is accumulating evidence to suggest that intact cells of *A. salmonicida* remain in aquatic habitats after the number of colony-forming units has declined to zero[17]. Attempts to revive these cells, principally by the addition of

nutrients, have been mostly unsuccessful. Whether the cells are damaged or dying has not been resolved but it is possible that they are present in an altered condition, necessitating specialized growth conditions. This is supported by studies by Effendi and Austin[18] of marine samples considered to be devoid of culturable *A. salmonicida* but that contained cells capable of passing through the pores of 0.22 and 0.45 μm porosity filters; some of these cells grew on specialized media developed for the growth of cell-wall-defective/deficient forms of *A. salmonicida*, i.e. L-forms[19]. Thus, populations of *A. salmonicida* of ~10^3 cells ml^{-1} were recorded in experimental microcosms after conventional plate counts had reached zero. These findings suggest that specialized forms of *A. salmonicida*, such as L-forms, may contribute to the difficulty in recovering the pathogen from environmental samples. Yet, the role of such modified cells in disease outbreaks is unclear. Certainly, natural L-forms have been observed in salmonids but conclusive proof of an association with disease outbreaks is lacking[19]. If *A. salmonicida* cells that are dormant, non-culturable, altered, senescent or dying are unable to reproduce disease then their relevance to fish pathology is questionable. It has been realized that nucleic acids from *A. salmonicida* may be shed into the aquatic environment. In particular, DNA has been found in aquatic sediments >13 weeks after colony counts declined to below detectable limits[20]. In future, molecular biology techniques may well be used to address the issue of the presence of living versus dead cells, principally by the detection of specific RNAs that should be present in truly viable cells.

Transmission

Fish are undoubtedly important in the transmission of furunculosis. Indeed, clinically diseased and carrier fish have long been associated with the spread of infection[1]. Carriers harbour the pathogen in the external mucus, gills and spleen[6], enabling release when clinical disease occurs, such as after immunosuppression[1]. Therefore, effective methods are essential for the detection of carriers. A combination of increasing the water temperature to 18°C and the injection of corticosteroids is often employed to induce the development of clinical disease from carriers[1].

Antibiotic therapy, which is often used to combat outbreaks of furunculosis[1], does not necessarily eliminate the carrier state. Indeed, it is possible that inappropriate antibiotic treatment regimes might lead to the establishment of altered forms of *A. salmonicida*, such as L-forms, in fish.

Certainly, contact with infected fish or contaminated water must be regarded as the most likely means for the transmission of furunculosis[1]. The site(s) of uptake of the pathogen into fish, although remaining the subject of conjecture, seems likely to include gills, mouth, anus and/or surface injury. By immunofluorescence, Klontz[21] determined that the intestine is the primary site of infection, leading to the development of the asymptomatic carrier state. McCarthy[22] showed that rainbow trout (*Oncorhynchus mykiss*) resisted infection with *A. salmonicida* unless the flanks were abraded. This suggests that *A. salmonicida* may enter through damaged areas on the surface of the fish. Further work demonstrated that uptake was enhanced by the presence of particulates, leading to the presence of the pathogen in the blood within a few minutes[23].

Control

Because of the economic importance of salmonid farming, much emphasis has been placed on developing effective control strategies for furunculosis. Methods have involved use of good husbandry practices (including 'good' water quality, adequate disinfection of equipment and eggs, and lower stocking densities), and disease-resistant fish stock, improved diets, nonspecific immunostimulants, antimicrobial compounds, probiotics (microorganisms that exert a beneficial effect on the host) and vaccines. Recent research has led to the recognition of the value of immunostimulatory compounds such as β-1,3-glucans, synthetic peptides and killed mycobacterial cells, the latter of which enhanced disease resistance in coho salmon to *A. salmonicida*[24]. Commercial interest has centred on β-1,3-glucans for control of infections by *A. salmonicida* when administered by injection[25]. β-1,3-Glucans are now being routinely incorporated into fish diets but this possibly reflects a misrepresentation of scientific data for commercial gain; specifically, the scientific evidence pointed to the benefit of glucans as nonspecific immunostimulants when administered by injection, not orally. It remains for further work to establish the effect of β-1,3-glucans on fish when administered orally.

Vaccine research has developed since the work in 1942 of Duff[26], who used a chloroform-inactivated whole-cell suspension of *A. salmonicida*, and encompasses use of subcellular components (namely inactivated extracellular products and lipopolysaccharides), genetic manipulation of cells and live (avirulent) vaccines with or without adjuvants. Iron-regulated outer-membrane proteins, the so-called IROMPs, are especially immunogenic to Atlantic salmon[27], and have been developed into a commercial vaccine. This product is now extensively used in the UK, and may be responsible for the marked reduction in the incidence of furunculosis in farmed Atlantic salmon since 1992. Yet, in laboratory-based studies, an IROMP-based vaccine was less successful than a product containing inactivated L-forms of *A. salmonicida*[28]. An outer-membrane porin has also shown potential in vaccine trials[29]. A significant development is an *in vivo* growth model for *A. salmonicida*, i.e. a specialized intraperitoneal chamber implanted in rainbow trout, which has allowed the prospect of developing gene products that are only synthesized in the host[30,31]. Genetic manipulation of *A. salmonicida* has led to the development of a subunit vaccine, through expression of a 587 kbp fragment of the 70 kDa serine protease gene in *Escherichia coli*[32]. Avirulent cells lacking the extracellular A-layer (this is involved in pathogenicity) have

been proposed as suitable vaccines[32]. An attenuated live vaccine (DELTA-aroA) has been developed, and preferentially stimulated T-cell responses in rainbow trout[33]. Here, an *aroA* mutant (BRIVAX 1) of *A. salmonicida* was constructed, and found to elicit antibody response. In addition, a live attenuated vaccine has been used as a carrier for heterologous antigen expression[34]. Certainly, the administration of such vaccines to fish led to the development of antibodies specific for *A. salmonicida*[35]. Following a comparison of intraperitoneal, immersion and oral vaccination methods in Atlantic salmon, the benefit of the first method was clearly demonstrated in terms of the highest antibody titre and level of protection[35]. Within 16 weeks of administration by injection, *A. salmonicida* lipopolysaccharide was located mostly in the abdominal granulomas, head kidney and spleen[35]. The benefit of adjuvants, especially those comprising mineral oil, for stimulating a protective immune response following intraperitoneal vaccination has been confirmed[36], despite the potential harmful side-effects to fish. Studies are now being directed at developing improved, non-oily adjuvants, which have negligible harmful effects on fish. In addition, an effective oral vaccine is sorely needed by the aquaculture industry.

Members of the normal microflora of fish may be useful as probiotics. Already, a strain of *Vibrio alginolyticus*, which was recovered from penaeid shrimps, has been found to aid the control of furunculosis in salmonids[37]. Other probiotics that may control furunculosis will undoubtedly be identified in the future.

Conclusion

Biotechnology is involved in the detection and control of infections caused by *A. salmonicida*. Clearly, there is an urgent need for rapid and reliable diagnostic systems suitable for field use. Such systems should provide information about the presence or likely onset of a disease condition, and not generate data on possible 'natural' population levels of *A. salmonicida*. In addition, disease control measures will continue to be researched, particularly concerning probiotics, non-specific immunostimulants and vaccines. A pessimistic view is that infections caused by *A. salmonicida* will not disappear from farmed fish populations, necessitating the continual development of 'new' or improved disease control measures.

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In the next issue: Bioremediation of marine oil spills

Controlled overproduction of proteins by lactic acid bacteria

Oscar P. Kuipers, Pascalle G. G. A. de Ruyter, Michiel Kleerebezem and Willem M. de Vos

Lactic acid bacteria are widely used in industrial food fermentations, contributing to flavour, texture and preservation of the fermented products. Here we describe recent advances in the development of controlled gene expression systems, which allow the regulated overproduction of any desirable protein by lactic acid bacteria. Some systems benefit from the fact that the expression vectors, marker genes and inducing factors can be used directly in food applications since they are all derived from food-grade lactic acid bacteria. These systems have also been employed for the development of autolytic bacteria, suitable for various industrial applications.

In the past few decades, numerous genetic tools have been developed for lactic acid bacteria (LAB), Gram-positive bacteria that are used in industrial food fermentations. These include the development of transformation, transduction and conjugation procedures[1], as well as the isolation and characterization of plasmid-free strains, stable cloning vectors, efficient promoters and the functional analysis of a plethora of genes encoding relevant proteins[2–4], any number of which could improve the properties of fermented products upon overproduction. Most advances in these areas have been made with strains of *Lactococcus lactis* that are widely used as starter cultures for cheese and other dairy products[1–4].

A desirable feature of the genetic tools that are being developed to modify LAB is their suitability for safe application in food. Ideally, this means that a so-called food-grade cloning host is a microorganism with a long history of safe use in food, which is currently true for all lactic acid bacteria. These hosts should contain plasmids or other DNA elements (e.g. transposons, bacteriophage DNA) that do not harbour any undesired genes, such as those coding for antibiotic-resistance markers. When use is made of an inducing factor for gene expression, this factor should be acceptable in food products, for example small inorganic molecules like salts, organic molecules such as saccharides or fatty acids, or proteinaceous compounds originating from LAB. Alternatively, changes in growth conditions, including pH, temperature, aeration or even phage infection, would be an acceptable way to induce certain activities. For some applications, expression systems should consist of elements that all originate from a single LAB species, since this results in strains improved by self-cloning. Several systems for self-cloning have been described, including one that uses a complementing *lacF* gene as a selectable marker for growth on lactose[5] and a system based on complementation of nonsense mutants in the purine biosynthetic pathway using plasmids with an ochre suppressor as a selectable marker[6].

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Regulated gene expression systems for lactic acid bacteria

Various expression systems using stable, broad host-range vectors and constitutive promoters have been described to date for different LAB, e.g. lactococci, lactobacilli, streptococci and leuconostocs[1–4]. In addition, chromosomal integration of genes at desirable loci is commonly used in microorganisms that can be transformed with relatively high efficiencies[2]. Here we will focus exclusively on expression systems that are based on transformation of LAB with plasmids containing effective expression signals, which can be regulated by inducers, repressors or environmental factors. Clearly, there is a need for such regulated expression systems for LAB, since ideally they will allow the controllable overproduction of proteins at high levels at any desired moment during industrial fermentation. This also means that proteins that are deleterious to the cell can be produced, because only minute amounts of these proteins will be produced by cells in the uninduced state. Regulated gene expression also

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PII: S0167-7799(97)01029-9

Table 1. Characteristics of inducible expression systems for LAB

LAB	Inducible element	Inducing factor	Expressed gene(s)	Ratio induced:uninduced	Refs
L. lactis	*lacA* or *lacR* promoter	Lactose	*cat-86, luxAB*	<10	7,8
L. lactis	*lacA*/T7 promoter	Lactose	Gene for TTFC	<20	9
L. lactis	*dnaJ* promoter	High temperature	*amyS*	<4	10
L. lactis	*sodA* promoter	Aeration	*lacZ*	2	11
L. lactis	*prtP* or *prtM* promoter	Absence of peptides	*gusA*	<8	12,13
L. lactis	repressor/operator ϕr1t	Mitomycin C	*lacZ*	70	14
Lb. pentosus	*xylA* promoter	Xylose	*cat-86*	60–80	15
L. lactis	PA170 promoter	Low pH, low temperature	*lacZ*	50–100	17
L. lactis	*trpE* promoter	Absence of tryptophan	*lacZ*	100	16
L. lactis	ϕ31 promoter and *ori*	ϕ31 infection	*lacZ*	>1000	18
L. lactis	*nisA* or *nisF* promoter	Nisin	*gusA, pepN, lytHA, nox estA, ptsH, pepO, ccpA*	>1000	19–21

provides the opportunity to study the effects of varying intracellular levels of protein production on metabolic pathways, provided that the level of expression is correlated with the amount of inducer added. Several studies on regulated gene expression, mainly in *L. lactis*, have been described, and some of these will be discussed here (Table 1).

The lacA*-promoter based systems*

One of the first examples of regulated gene expression was described for the *lacA* promoter of the lactose operon of *L. lactis*[7]. Fragments containing the *lacA* promoter and increasing lengths of flanking regions were fused to the promoterless *cat-86* gene. It was shown that expression was dependent on the LacR repressor, being derepressed during growth on lactose by the formation of the inducer tagatose-6-phosphate. Sequences downstream and upstream significantly enhanced promoter activity (R. J. van Rooijen, PhD Thesis, University of Wageningen, 1993). Protein engineering studies with LacR yielded mutant repressors with improved activity. The level of *cat-86* expression in cells grown in lactose was 5–10-fold greater than in those grown in glucose (Ref. 7; R. J. van Rooijen, PhD Thesis). The *lacA* and *lacR* promoter activities were also studied by Eaton *et al.*[8], who reported a 5- and 7-fold induction of *lacA* and *lacR* promoters, respectively, by growth on lactose, using the reporter genes *luxAB*. The lactose-inducible system always shows a significant background expression on glucose, hampering the production of 'lethal' proteins. Moreover, for industrial purposes, it is not very attractive to switch the sugar during large-scale fermentations.

An improvement on the use of the *lacA* promoter was realized by Wells *et al.*[9], who described the development of a lactococcal system that uses the T7-polymerase of *Escherichia coli* bacteriophage T7 to transcribe target genes. The polymerase gene itself is expressed under control of the *lacA* promoter, and induction takes place by growing the cells in lactose, during which a significant increase in expression level of the gene of interest cloned downstream of the T7-promoter was achieved. However, significant background expression of the gene of interest does occur. The system was applied to produce substantial amounts (22% of total intracellular protein) of heterologous tetanus toxin fragment C (TTFC), with the ultimate goal to use *L. lactis* as an antigen delivery vector for oral antigen administration. Promising results were obtained when it was demonstrated that the overexpressing strain could generate antibodies in mice, which subsequently protected them from a lethal TTFC challenge[9].

The dnaJ*-promoter based system*

Another type of gene regulation was found to occur in the case of the expression of the *dnaJ* gene of *L. lactis*, which could be induced by heat shock[10]. A transcriptional fusion between the *dnaJ*-promoter fragment and a signal sequence derived from the lactococcal *usp45* gene, followed by *amyS* encoding the *Bacillus stearothermophilus* α-amylase, resulted in a 3–4-fold increase in extracellular α-amylase activity upon raising the temperature from 30 to 42°C. The mechanism by which the induction is brought about is certainly interesting, however, the applicability to industrial fermentations of a *dnaJ*-promoter based system is limited, because of the relatively low level of induction, the high background expression level in the uninduced state and the undesirable shift of temperature, which will also affect expression levels of many other genes.

The sodA*-promoter based system*

Analysis of stress response in *L. lactis* revealed that the synthesis of at least three proteins is induced at low pH values of the culture medium[11]. The gene coding for one of these proteins was cloned and sequenced, and was shown to encode a superoxide dismutase. The expression of the *sodA* gene was analysed by transcriptional fusion with a promoterless *lacZ* gene. The β-galactosidase activity in aerated cultures of *L. lactis* was twice that of nonaerated cultures[11]. Regrettably, no induction experiments at low pH values or at different time points during growth were described.

Although the mechanism of regulation of gene expression by oxygen pressure is quite relevant for industrial fermentations, the induction level that can be obtained is too low for industrial protein production. However, this work provides an attractive tool for metabolic engineering, e.g. modulating enzyme activities in *L. lactis* by aeration during growth.

The prtP*-promoter based system*

Two genes are involved in the production of the cell-envelope-associated proteinase of *L. lactis*, i.e. *prtM* and *prtP*, encoding a maturation protein and the protease, respectively. It has been shown that transcription of these divergently transcribed genes is controlled in an identical manner, and is dependent on the peptide concentration in the medium[12,13]. Transcriptional fusions of these promoters with *gusA* showed that the activity was depressed at high peptide concentrations, particularly by the dipeptides prolylleucine and leucylproline, whereas the level of expression was about eightfold higher in media that contained relatively low amounts of peptides. Although these findings are relevant for the control of industrial fermentation processes in relation to growth characteristics, the system itself might not have a high potential for regulated gene expression of other genes, because of the relatively low inducibility and the rather difficult task of controlling peptide concentrations during bacterial growth.

The repressor/operator of ϕr1t based system

A regulated gene expression system employing somewhat different tools was recently developed using the repressor–operator system from the completely sequenced *L. lactis* bacteriophage r1t (Ref. 14). This phage contains a DNA fragment with a gene, *rro*, that specifies a protein that represses gene expression from a promoter on the same fragment. This region was exploited for inducible gene expression in *L. lactis* by transcriptional fusion to the *lacZ* gene. By applying conditions that normally induce the switch from the lysogenic to the lytic life cycle of r1t, i.e. by the addition of the DNA-damaging agent mitomycin C, a 70-fold increase in β-galactosidase activity was obtained. Obviously, this system holds potential for overproduction of proteins by *L. lactis*, if another, more food-acceptable, induction system can be applied. This could be based on a thermosensitive repressor as has been suggested[14], although many large-scale processes do not easily permit the use of thermal switches during production.

The xylA*-promoter based system*

The *xyl* genes of *Lactobacillus pentosus*, involved in xylose catabolism, are induced by xylose and repressed by glucose, ribose and arabinose[15]. Both the repressor XylR and the mechanism of catabolite repression are responsible for the reduced expression on glucose. Fusions have been made between the *xylA* promoter fragment and the promoterless *cat-86* gene and these were transformed into *Lactobacillus pentosus*. Growth on xylose resulted in a 60–80-fold higher CAT activity than growth on glucose[15]. This system provides a valuable tool for controlled gene expression in *L. pentosus*. However, switching to xylose as a carbon and energy source during industrial fermentations is less attractive for economic reasons.

The tryptophan controlled system

Transcriptional studies on the regulation of the tryptophan (Trp) biosynthetic genes *trpEGDCFBA* of *L. lactis* showed that the *trp* genes are organized in a single operon and that the synthesis of this transcript is regulated by a factor of about 100 by Trp, through the mechanism of transcription termination[16]. Other expression studies using transcriptional fusions between the *trp* promoter and the *lacZ* gene showed that β-galactosidase activity was negatively affected by Trp (Ref. 16). This interesting system could be valuable for laboratory applications to express genes, but probably has limited value for biotechnological applications in view of the difficult and expensive task of modulating the Trp concentrations in commercial media used for fermentation.

The PA170-promoter based system

Transposon insertions that result in fusions of different chromosomal loci of *L. lactis* to a promoterless *lacZ* gene have been reported to yield hundreds of β-galactosidase-producing integrants[17]. Several of the integrants were analysed for regulated gene expression and one of these, PA170, was shown to produce considerably more β-galactosidase under different conditions. The β-galactosidase activity was higher when cells were grown at pH 5.2 than at pH 7.0, higher at 15°C than at 30°C and higher in stationary phase than in logarithmic phase. A 9.7 kb DNA fragment from PA170 was cloned on a multicopy vector and introduced into *L. lactis*. This strain showed a similar mode of regulation; differences in induction levels ranged from 8–50-fold, depending on the conditions used[17]. This property might be valuable for the construction of genetically improved strains for industrial applications, since the regulation takes place through naturally occurring processes; for example, during cheese ripening. Further characterization and engineering of this promoter region might improve the induction factor and the final level of expression. In addition, a combined effect of low temperature and low pH could be expected to yield tighter control of gene expression.

The ϕ31-middle-promoter and ori *based system*

An original strategy for regulated gene expression in *L. lactis* has been based on two combined features of a lytic bacteriophage[18]. Plasmids were constructed that contained the *lacZ* reporter gene under control of a ϕ31-promoter region. After introduction into *L. lactis*, the resulting strain was infected by ϕ31, resulting in considerable overproduction of β-galactosidase. A further improvement of this system was achieved by introduction of the phage origin of replication on

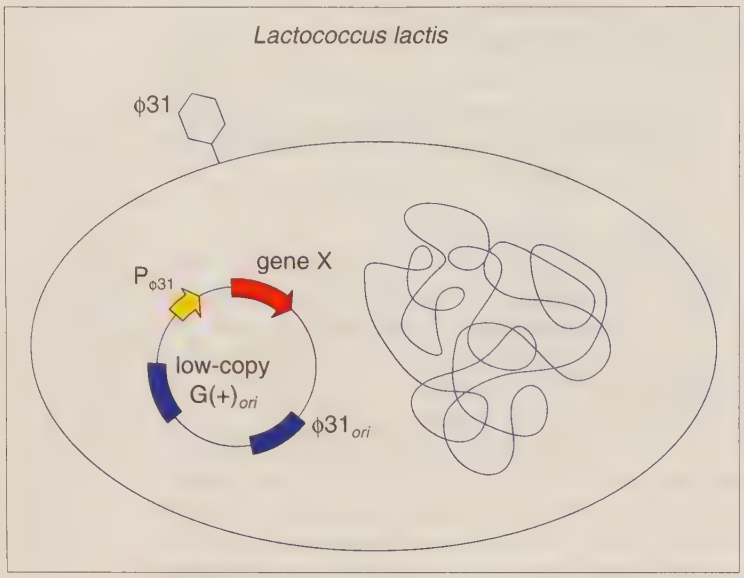

Figure 1
Sequence of events following ϕ31 infection of lactococcal cells harbouring the plasmid for explosive gene expression (Ref. 18). 0 min: Induction by ϕ31 infection; 20 min: effective plasmid amplification reached; 40 min: high gene transcription/translation reached; 60 min: lysis and release of overproduced proteins.

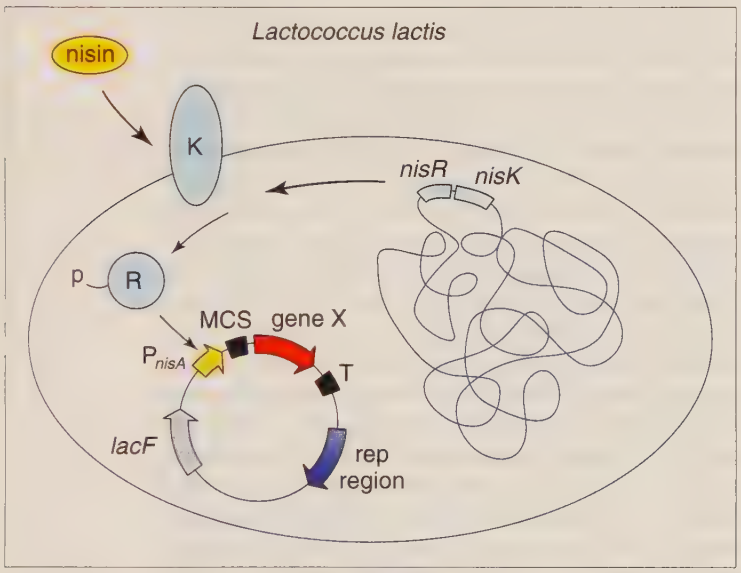

Figure 2
Model for nisin signal transduction involving the sensor histidine-kinase NisK and the response regulator NisR (Refs 19 and 22–25). T: Transcriptional teminator.

a low-copy number expression plasmid, to allow phage-inducible plasmid amplification. The expression vector is maintained at low-copy number in the host, allowing the cloning of genes encoding toxic proteins. After a deliberate phage infection, gene expression was 'explosively' triggered within 60 min, resulting in at least a 1000-fold increase in β-galactosidase activity[18] (Fig. 1). This illustrates the power of such a system to produce high amounts of protein in a relatively short time. The proteins can be maintained within the host if the cells are harvested prior to lysis, or allowed to be released into the medium if lysis is permitted. This expression system can, in principle, be constructed in a food-grade manner. A drawback for broader application might be that modulation of gene expression seems to be hard to achieve with this system.

The nisA/nisF*-promoter based system*

A new system that offers significant potential for regulated gene expression in lactic acid bacteria is based on signal transduction by the two-component regulatory system, consisting of the response-regulator protein NisR and the sensor histidine-kinase NisK, found in the nisin gene cluster of *L. lactis*. Nisin is a 3.5 kDa highly modified antimicrobial peptide, which is widely applied as a food preservative. Extracellular nisin acts as the signal for transcriptional activation of its own structural gene and of several other biosynthetic and immunity genes[19–21]. A model of the signal transduction pathway of the nisin genes, based on the general characteristics of two-component regulatory systems[19,22–25], is presented in Fig. 2. When plasmids with genes under control of the *nisA* promoter fragment are introduced in strains that cannot produce nisin but do contain the NisR and NisK proteins, which are necessary for signal transduction, these genes will be expressed at a very low level. This expression level is in most cases undetectable, even when using sensitive assays based on β-glucuronidase production. When nisin is added to the growth medium during logarithmic growth, transcription of the genes that are controlled by the *nisA* promoter is activated. The level of expression is proportional to the amount of nisin added[19]. Recently, it was found that the *nisF* promoter, driving expression of the *nisFEG* genes, which were shown to be involved in immunity to nisin[26,27], can be activated by nisin in a way similar to the *nisA* promoter[20]. To date, this system has been applied to express several homologous and heterologous genes, e.g. *gusA* of *E. coli*, *pepN* (Ref. 28) of *L. lactis*, and *lytH* and *lytA* (Ref. 29) of ϕUS3 of *L. lactis*. A series of expression strains and vectors has been developed that allows high nisin-sensitivity of the signal transduction pathway and high production of the proteins of interest, e.g. by construction of vectors especially suited for making translational fusions at the start codon of *nisA*, thereby employing the strong translation-initiation signals of this gene[19,21]. Examples of the efficiency of the expression system are shown for *gusA* and *pepN* expression in Fig. 3. Levels of intracellular production of different proteins typically range between 10 and 60% of total soluble protein.

The nisin-inducible system has several advantages for application:

- Nisin is an attractive molecule for use as an inducer, as it already has a history of safe use in food products and therefore can be considered food-grade.
- The system is easy to use and low-cost, as induction of cultures can take place by simply adding small amounts of nisin [typically between 0.05 and 5 μg(nisin) l^{-1}, or less than 0.1% of a nisin-producing *L. lactis* fermentation in milk]. This means that other

starter culture bacteria will not suffer any damage from these subinhibitory amounts of nisin. With some nisin mutants even lower concentrations are required for induction than with wild-type nisin[19].

- It is a versatile and flexible system because several different expression strains and plasmids, containing convenient multiple cloning sites, are currently available[21].
- It seems feasible to implement this system in LAB other than *L. lactis*, by introducing *nisRK* genes on a plasmid and other plasmids containing the *nisA* promoter and the gene(s) of interest.
- The expression is extremely tightly controlled, leading to essentially no detectable production of the protein of interest in the uninduced state, enabling production of lethal proteins.
- A controllable level of expression can be obtained, depending on the amount of nisin added, leading ultimately to very high protein production, of up to 60% of total intracellular protein. The cells will in most cases not lyse, so it can be anticipated that secretion of proteins using suitable signal sequences can be achieved, facilitating downstream processing.
- A fully food-grade system has been obtained by use of *lacF*-deficient lactococcal strains[5] that have *nisRK* genes integrated on the chromosome, complemented by plasmids containing the *nisA* promoter controlling expression of the gene of interest, the *lacF* gene as a selectable marker and nisin as an inducer[21].

Examples and prospects of application of controlled expression systems

For industrial enzyme production the ϕ31-promoter/*ori*- and *nisA*-promoter based systems probably hold the most potential in view of their ability to produce very high amounts of protein. The ϕ31 based system has the additional advantage that it reaches high production in less than an hour after phage infection. For *in situ* production of enzymes by starter cultures or for metabolic pathway studies, the nisin-inducible system is ideally suited since any desired production level less than maximum can also be obtained. Another interesting application of the nisin-inducible system could be the regulated production of antisense RNA, enabling modulation of enzyme activities during growth of the bacterium.

Recently it has been reported that non-modified peptides encoded in some bacteriocin gene clusters of LAB other than lactococci can also have a signalling function and activate transcription of specific genes via signal transduction[30–32]. Although no examples of controlled overproduction of proteins using these inducing peptides, putative signal transduction proteins and promoter elements have been reported yet, systems based on these elements certainly hold potential for future application.

Finally, food-grade inducible expression systems have a high potential for developing autolytic starter cultures, which could accelerate cheese ripening. An example of an inducible autolytic bacterium was reported by Shearman *et al.*, who used induced expression of two lytic genes of ϕvML3 by putting them under the control of a T7-promoter fragment from bacteriophage T7 of *E. coli*, yielding a 34-fold increase in lysin activity in *E. coli* after induction with IPTG (Ref. 33). However, no controlled expression in LAB was achieved due to the lack of a suitable controlled expression system. Recently, de Ruyter *et al.*[21] fused the holin-lysin encoding cassette *lytHA* of ϕUS3 to the *nisA* promoter fragment, which yielded a stable construct in *L. lactis*. The lytic muramidase LytA can degrade cell walls of LAB and is thought to be exported by passive diffusion through pores formed by the holin protein LytH. Upon induction of the construct by addition of nisin to the growing culture a rapid and efficient lysis of the cells occurred. This result illustrates the applicability of tightly controlled expression systems for the expression of lethal genes. An attractive possibility for application of this system is to

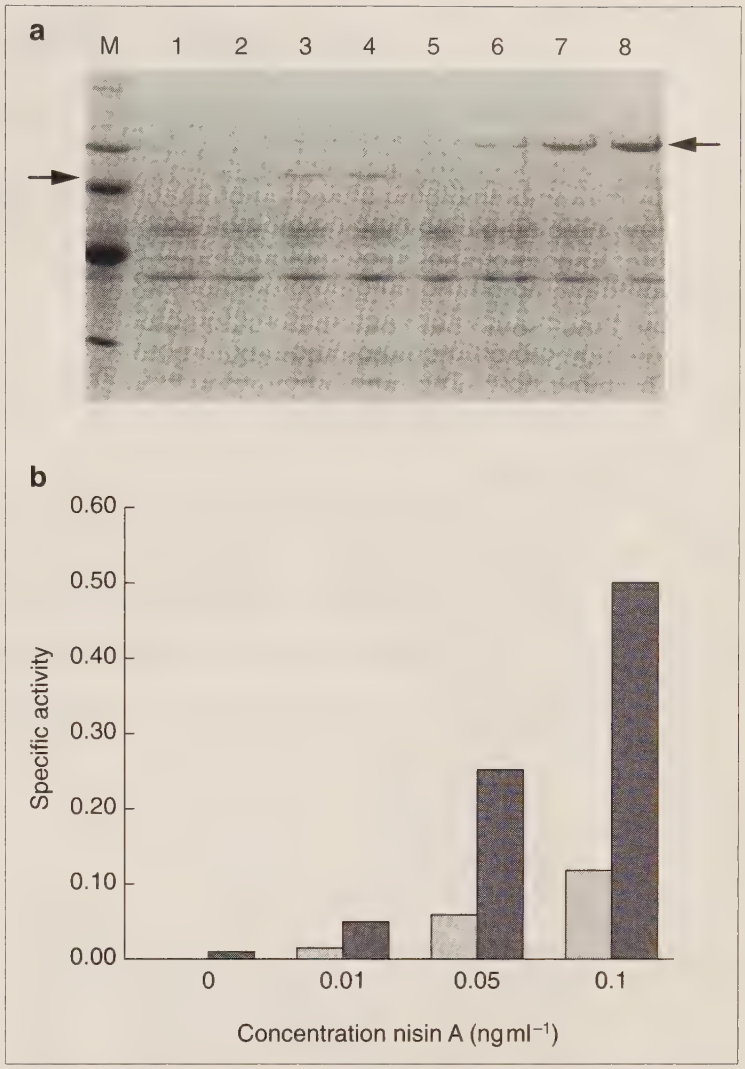

Figure 3

(**a**) Coomassie blue-stained gel after SDS–PAGE of extracts of *L. lactis* containing the *PnisA-gusA* and the *PnisA-pepN* fusion plasmids producing β-glucuronidase (lanes 1–4), or aminopeptidase N (lanes 5–8), after induction with nisin. Lanes 1–4: 0, 0.1, 1 and 5 ng(nisin A) ml^{-1} added, respectively. Lanes 5–8: 0, 0.05, 0.1 and 1 ng(nisin A) ml^{-1} added, respectively. (**b**) β-Glucuronidase (light grey) and aminopeptidase N (dark grey) specific activities shown in AU per $A_{600\ nm}$.

combine expression of the lytic genes with that of genes involved in flavour formation, e.g. peptidases, lipases and amino-acid convertases. Autolytic strains can also conveniently be used for controlled overproduction of desired proteins in the culture medium as an alternative to *sec*-dependent secretion systems. Future work will focus on assessing the effects of controlled lysis of LAB, combined with production of desirable enzymes, on the maturation of fermented products.

Acknowledgements

We are grateful to R. Jack and R. Siezen for critically reading the manuscript.

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Approaches to evaluating the transgenic status of transformed plants

James C. Register III

Analysis of transgenic plants is used today to answer the same questions as ten years ago. Since then, a wide variety of new molecular and biochemical technologies, as well as genetic tools, have become available. While there are many technical manuals describing how to use these tools, much less has been written about when they should and should not, be employed. This gap is important, as poor analysis can easily sidetrack or derail a project. This review addresses appropriate and inappropriate applications of several technologies commonly used for molecular and biochemical analysis of transformed plants.

The field of plant genetic engineering has evolved rapidly over the past decade. Despite the extent of this change, the need to determine whether or not transformed material is transgenic remains. While this issue may seem straightforward, the frequency with which insufficient or inappropriate data are used to support claims of transformation demonstrates that obtaining clear answers is not as trivial as it might seem. Although there are numerous manuals detailing how to carry out essentially any molecular technique, far less attention has been paid to describing the types of analytical approaches that provide appropriate data for answering a variety of commonly asked questions, including whether transformation of a plant has been achieved. This review will address appropriate and inappropriate applications of several technologies commonly used for molecular and biochemical analysis of nucleic acids and proteins in transformed plants.

Definitions

Before proceeding, a few definitions should be established for the purposes of this review. Transform(ed) will be used to refer to the act of introducing DNA into plant cells as well as to plant cells after DNA introduction. Transgenic will not be used interchangeably with transformed but will be used to refer to plant cells in which the DNA of interest (the transgene) is integrated into the host genome. Regenerated transgenic plants will be referred to as the T_0 generation. Plant transcription unit (PTU) refers to all the vector components that contribute to transgene expression (i.e. promoter, coding sequence, polyadenylation site and any other regulatory sequences employed). Because it is not uncommon for several copies of a transgene to insert at the same site (regardless of the transformation approach; e.g. Refs 1–4), copy number and number of insertion sites of a transgene are not the same and these terms will not be used interchangeably.

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Analytical approaches

Molecular and biochemical analysis of transformed plant material is done for two reasons. First, to determine whether the material is transgenic and, second, to characterize the material (e.g. determine copy number and/or the complexity of the DNA insert, and evaluate transgene expression). A wide variety of analytical tools are available for routine transgenic plant analysis. Some of the commonly used technologies and applications are listed in Table 1.

Transgene presence and structure

Polymerase chain reaction

PCR technology (for reviews see Refs 5,6) is perhaps the most popular method for screening material for the presence or absence of transgene sequences. It is particularly useful for rapid analysis of large numbers of samples. Despite its popularity, PCR has several inherent characteristics that limit the conclusions that can be drawn. First, a positive PCR result shows only that sequences homologous to the primers exist in a sample in close enough proximity for a product to be made. The results do not indicate whether the template DNA source is the intended sample material or

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PII: S0167-7799(97)01008-1

Table 1. Commonly used analytical technologies and appropriate applications

Technique	Applications
PCR	Rapid screening of transformants to focus subsequent analysis Identifying transgenics in segregating populations
Quantitative PCR	Estimating copy number (total) Hemizygote/homozygote discrimination (potentially)
Southern hybridization	Screening for presence/absence of specific sequences Assessing complexity of integration/transgene rearrangements Estimating copy number (rearranged and unrearranged) Estimating whether transgenes inserted at single or multiple loci 'Fingerprinting' identity of independent events
Reverse transcription PCR	Transgene expression: accumulation of specific mRNA – can be (semi-)quantitative and used for determining relative levels
Northern hybridization[a]	Transgene expression: quantifying specific mRNA accumulation Estimating mRNA size
ELISA[b]	Transgene expression: rapid screening for presence/absence of specific protein Can be very high throughput Quantifying specific protein accumulation
Western immunoblot	Transgene expression: screening for presence/absence of specific protein Approximate quantitation of specific protein Estimating protein size, evaluating protein processing

[a]The ribonuclease protection assay can be used in many cases to obtain analogous results. Compared with northern hybridization, it is generally regarded as more sensitive and offers the potential for greater throughput but it cannot be used for estimating RNA sizes.
[b]Western immunoassays of protein dot blots can provide some of the advantages of an ELISA as compared with traditional western immunoassays of electrophoresed proteins (e.g. increased throughput), potentially with reduced assay development requirements.

due to contamination, nor whether the template DNA is integrated into the plant genome. Information about the sequence outside the primers is also not provided. When products are analyzed using electrophoresis, verification of product size can be made. Southern analysis of the products can confirm that the sequence in between the primers is as expected, but this step is time consuming and thus rarely carried out. Quantitative PCR (for reviews see Refs 7,8) opens up the opportunity to use this technology for providing information historically obtained using Southern hybridization, such as estimates of copy number. The requirements for synthesizing standards, and use of additional standards and controls[9,10], as well as the fact that Southern hybridization analysis provides some information that PCR does not, have kept this approach from being used routinely.

PCR as used for transgenic plant analysis has different requirements than PCR used for cloning. Foremost is the robustness required of the reactions – a 10% failure rate is unacceptable for analytical purposes. Several steps, in addition to those routinely recommended for all PCR applications, can be taken to maximize robustness when setting up and monitoring reactions during analysis. For example, keeping reaction products less than ~1000 bp in length, using relatively long primers (25–30 nucleotides) and using primers with a high T_m (~65–68°C) can help ensure robustness and specificity. Ensuring that primers are sufficiently sensitive for reproducible detection of single copy sequences is crucial, and primer location affects the utility of the information provided by PCR (Fig. 1). Finally, during analysis, amplification of a known endogenous single copy gene, in addition to the transgene, provides a useful control for monitoring template DNA quality.

The extreme sensitivity of PCR results in the potential for contamination and false positive results. A variety of approaches to minimizing PCR contamination, both physical and biochemical, have been described[5,11–13]. In my experience, attention to primer design (Fig. 1), experimental design and logistics (how and where the work is carried out) is sufficient to minimize the occurrence of false positive results.

Southern hybridization

Southern analysis (for reviews see Refs 5,14,15) is one of the more powerful tools available for molecular characterization of transgenic plants. Depending

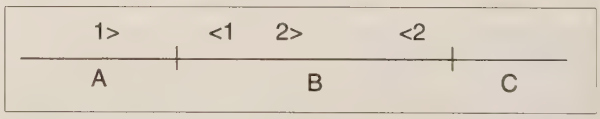

Figure 1

PCR primer location. The drawing shows a generic plant transcription unit in which A, B and C represent the promoter, coding sequence and polyadenylation site, respectively. They may also be thought of as any other juxtaposed DNA sequences in a vector. In this example, primers (1> and <1) are shown flanking a junction between DNA sequences not normally found in nature. This approach has two advantages over locating both primers within a single stretch of DNA (such as shown for primers 2> and <2): some information about the presence or absence of promoter, as well as the coding sequence, is provided and it may help avoid the occurrence of false positive results.

on how restriction digests are designed, information regarding the complexity of transgene insertion(s), the number of transgene copies present, the integrative status and the number of chromosomal sites where the transgene(s) has inserted can be obtained.

For analysis of copy number and integration complexity, digests should be designed to excise the entire DNA fragment for which information is desired. Most often, this will be a PTU, since it contains both the coding sequence and accompanying regulatory sequences. While it is not uncommon in the literature to see only the coding sequence analyzed, this approach does not provide as much information as analysis of the entire PTU. This approach can be used whether *Agrobacterium* or direct DNA delivery (DDD, e.g. electroporation or particle gun bombardment) was used to produce the material being analyzed. The vector DNA used for transformation should be digested and analyzed alongside sample genomic DNA to provide the necessary controls for evaluating rearrangements and copy number. Sample bands of a different size from the control band are considered rearranged; however, this analysis does not characterize the rearrangement(s). Copy number reconstructions can be made by diluting vector DNA to appropriate concentrations in a matrix of DNA from untransformed plants. Hybridizing signal strengths of these controls can then be compared with those in samples to estimate copy number. Because of the sample-to-sample variability between DNA preparations (with regard to degradation and digestibility) and the imprecision of genome size estimates (a review of the literature indicates up to a threefold spread in estimates for some species; compare for instance Refs 16,17), only rough estimates (± approximately 2–3-fold) can be made using this approach. Accuracy can be improved somewhat by also probing for an endogenous gene having a known copy number[18], but because this is more time consuming, and many labs do not have access to such probes, this approach is often not taken. Examples of the kinds of results that may be obtained from this analysis are shown in Fig. 2, lanes 5–8.

Southern analysis can be used to provide evidence for transgene integration, including insertion at multiple sites, although misleading results can be obtained. The analysis is most straightforward when the material being analyzed was produced using *Agrobacterium*. In this case the T-DNA right border typically provides a defined junction between vector and plant DNA which can be exploited as shown in Fig. 3a. In this border analysis, the presence of multiple bands provides evidence for multiple insertion sites, but not proof, as multiple bands can also result from transgene rearrangement and multimerization at single insertion sites. The situation is less straightforward for material produced by DDD as vector–plant DNA junctions are more random. Two approaches can be taken (Figs 3b and 3c). In one (Fig. 3b) the restriction enzyme cuts only within the plant DNA and not the vector used for transformation. The presence of multiple DNA bands is diagnostic for integration at multiple sites. Because the resulting transgene-containing fragments can be fairly large (particularly if the DNA used for transformation was larger than approximately 5–8 kb), multiple fragments may not be resolved using standard agarose electrophoresis conditions. An alternative is to use a restriction enzyme that cuts once within the transgene (Fig. 3c). This approach tends to produce smaller transgene-containing fragments but multiple bands due to transgene rearrangements and multimerization at single insertion sites can also occur. Examples of the kinds of results that can be obtained using these approaches are shown in Fig. 2, lanes 9–12.

In the approaches described above, a sample DNA fragment size different from that produced by the vector (particularly a higher molecular mass band) is indicative of, but does not prove, integration. Similarly, transgene probe hybridization to unrestricted high molecular mass DNA provides evidence for, but not proof of, transgene integration. Additional evidence, such as mendelian inheritance or cloned vector–plant DNA junctions, is required to demonstrate integration unambiguously. The importance of assessing the transgenic status of material using several complementary methods when the transformation approach being used is not well characterized has been illustrated by Langridge *et al.*[19].

Southern analysis of transgenes can be particularly problematic when PTU sequences are present in the untransformed host genome. In some cases this problem can be circumvented by using a probe to a part of the PTU not present in the genome, but this approach is not always feasible. In cases where the probe hybridizes to endogenous, as well as transgene, DNA, three precautions are recommended. First, try and use restriction enzymes that produce as simple a pattern as possible when the untransformed DNA is analyzed. Second, these fragment size(s) should be sufficiently different from those of the transgene fragment such that interpretation of the band pattern in transformed material is not ambiguous. Finally, untransformed genomic DNA controls need to be included on every row of a gel (this is always a good idea but is particularly crucial under these circumstances).

Transgene expression

Transgene expression studies typically examine either protein or RNA accumulation. While such analysis does a great deal to characterize transformants, the results do not directly address their transgenic status. Thus, correlating expression with stable transformation is inappropriate for all but the best-characterized transformation systems. The three questions asked most frequently in expression studies are: how much?, when? and where? The techniques described in this section can be used to provide detailed answers to how much? and when? and crude (i.e. resolution at the organ and sometimes tissue levels, but not at the cell level) answers to where? Experiments addressing how much? often require statistical analysis of data. Because transgene expression data from T_0 plants often do not have a normal distribution,

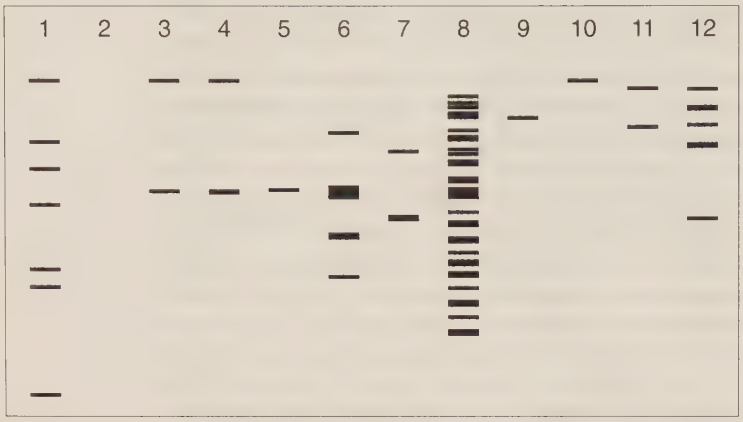

Figure 2

Representative Southern analysis results. This drawing shows cartoon examples of Southern data for either plant transcription unit (PTU) characterization (lanes 2–8) or analysis of the number of integration sites (lanes 9–12). Lane 1 represents a λ HindIII digest, which produces fragments of ~23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.6 kb. Lanes 2–4 represent copy number reconstructions for PTU analysis of a ~5 kb PTU, having 0, 1 and 5 copy equivalents, respectively. Lane 5 shows the expected result for a low (one to a very few) copy unrearranged PTU insertion. Lane 6 shows the expected result for material having both unrearranged (high copy number) and rearranged transgene copies. Lane 7 shows an example of a result from material having only rearranged transgene copies. Lane 8 shows an example of the extremely complex high copy number patterns that can occur, particularly with material produced using direct DNA delivery. Lanes 9 and 10 show examples of results from integration analyses that, as described in the text, indicate single insertion sites. In particular, the high molecular mass band in lane 10 may be misleading as multiple DNA fragments can co-migrate in this portion of the gel. Lanes 11 and 12 show patterns consistent with more than one insertion locus. As noted in the text, such results can also be misleading as, depending on the restriction digest approach used, these patterns (particularly as shown in lane 11) may result from rearrangement of multiple tandem copies. Patterns similar to that shown in lane 12 do correlate well with the presence of multiple insertion sites and are often seen with material having a PTU profile like that shown in lane 8.

statistical tools not typically employed by molecular biologists or biochemists must be considered[20]. Detailed tissue and cell localization studies require other approaches such as *in situ* hybridization (for reviews see Refs 21,22), which are beyond the scope of this review. Some marker genes also offer the potential for histological analysis (see below).

RNA analysis

In most cases, analysis of transgene expression focuses on proteins or other end products since the desired phenotype results from accumulation of a specific protein. When protein analysis is not possible, analysis of RNA transcripts is often used. Even in cases where protein analysis is carried out, RNA analysis can provide useful information about transcript accumulation and stability that may help explain unexpected phenotypes. Techniques such as reverse transcription (RT) PCR[23], northern analysis[14,15,24] and the ribonuclease protection assay (RPA; Ref. 25) can be used to measure steady-state RNA levels. Analysis of RNA synthesis or degradation rates requires nuclear transcription run-on analysis[26].

RT-PCR can be used as a quick and relatively high-throughput screen for the presence or absence of a specific transcript. The advantages of RT-PCR, as compared with northern analysis or RPA, are the small amount of material needed, the high sensitivity and throughput and the ease of sample preparation. Unlike northern analysis, however, RT-PCR results provide no information about transcript size. If RT-PCR is to be used for quantitative analysis, additional effort and controls are required[27,28]. The ability to discriminate between products resulting from amplification of sample genomic DNA versus cDNA synthesized from sample RNA *in vitro* is critical when using RT-PCR. Thorough DNase digestion of RNA preparations is always required and, whenever possible, primers should be located such that amplification of sample DNA is either impossible or results in a product having a different size than a cDNA amplification product[29,30].

Immunoassays

Immunoassays[31] are the most commonly used approach for studying accumulation of proteins in transgenic plants. There are two broad types of immunoassay typically employed: immunoblot hybridization (western) and the enzyme-linked immunosorbent assay (ELISA).

Western immunoassays and ELISAs have largely complementary strengths and weaknesses. Advantages offered by westerns over ELISAs include less assay development (ELISA development can be nearly as much art as science) and the ability to assess protein molecular masses. In addition, acceptable results can often be obtained using relatively impure antibody preparations. However, westerns are much more time consuming to run than are ELISAs and quantitative results are approximate. Historically, westerns were often less sensitive than ELISAs; however, with the appearance of robust chemiluminescent detection systems (e.g. Ref. 32) the magnitude of this difference has decreased. Indeed, the costs of assay development and adopting the quality assurance measures required to obtain the most accurate ELISA results (e.g. Refs 33,34) may more than offset the advantages for many applications.

Foremost among the technical problems typically encountered when using immunoassays is an unacceptable background[31,35,36]. Certain backgrounds may be due to the specific interaction of an antibody (either primary or secondary, if more than one antibody is used) with a protein in the extract, while others are nonspecific. The two most common sources of nonspecific background problems are antibody dilutions and blocking. Backgrounds can often be reduced or eliminated by decreasing antibody concentrations, and the optimal blocking reagent and blocking conditions should be determined empirically for each protein.

Transgene product activity

In most transgenic plants, the desired phenotype is due to a specific activity of the protein product. This activity may or may not be enzymatic and may be measurable using a biochemical assay and/or some

type of bioassay (e.g. resistance to an insect or pathogen pressure). In any event, it is important to remember that, since this activity is, in many cases, the most direct link to the desired *in planta* phenotype, its measurement may be crucial to understanding the phenotype. While the myriad of potential assays that could be used for such analysis cannot be covered adequately here, such analysis should be included as a part of a comprehensive molecular and biochemical analytical package. In an analogous manner, such analysis of endogenous enzyme activity can be useful when antisense (or sense) gene suppression is being attempted. It is important to remember that activity assays are not measuring the same thing as an immunoassay and so activity results will not necessarily correlate with protein accumulation levels.

Marker enzymes

A number of scorable marker enzymes have been used extensively in plants, most notably β-glucuronidase[37,38] and luciferase[39]. Recently, an autofluorescent protein from jellyfish, the green fluorescence protein, was described[40], and its use in plants seems certain to increase (e.g. Ref. 41). These markers are extremely useful in transgene expression studies (some for both quantitative and histochemical analyses), for promoter characterization and for quickly screening large numbers of transformants so that subsequent detailed analysis can focus on the most appropriate subset of plants.

The general utility of marker genes and the ease with which results can be obtained has unfortunately resulted in their use for inappropriate purposes. Specifically, expression of a marker gene, like expression of any other gene, does not necessarily correlate with stable transformation. When developing a transformation system, screening hundreds-to-thousands of putative transgenics is often required to identify that first rare event or to optimize the transformation system. In scenarios such as these, it makes sense to use a rapid high-throughput screen to select the material that deserves subsequent analysis and marker analysis, like PCR, provides a powerful tool for such screening. Problems arise, however, when positive results from such screening are used as verification of transgenic status. As described above, other technologies must be used to obtain this information.

Inheritance of transgenes

The same techniques and approaches described above for determining the transgenic status of transformed material can be applied to analysis of transgene inheritance. Just as transgenes can be stably integrated into a host plant genome without being expressed, giving rise to potential analytical problems, transgenes can be inherited in a predictable manner but not expressed (see Refs 4,42 and refs therein). Noninheritance as well as inheritance inconsistent with mendelian genetics can also occur (see Refs 4,42 and refs therein). Therefore, obtaining a complete picture of transgene inheritance may require use of multiple complementary molecular and biochemical approaches. The same suggestions and caveats presented earlier in this review regarding application of various techniques should also be considered when developing an approach for analysis of inheritance.

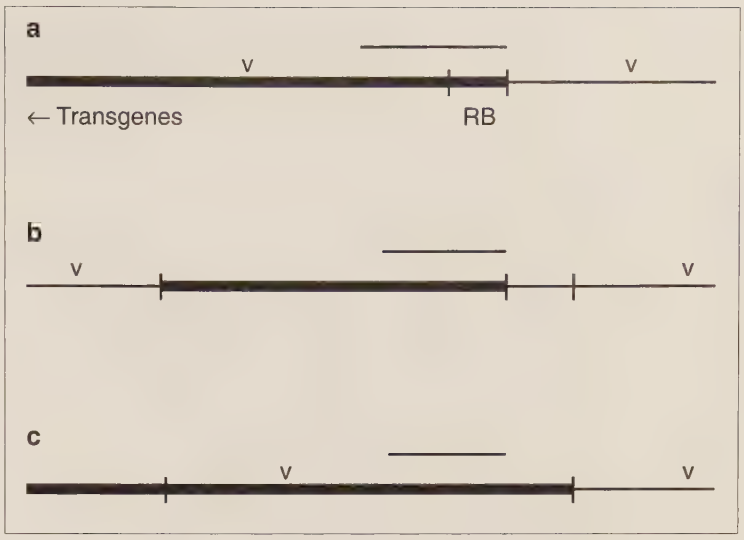

Figure 3

Southern analysis for determining the presence of multiple integration sites. The schematic diagrams represent vector (transgene) DNA (▬) integrated into plant DNA (—). Restriction sites are shown (v) and probe locations are indicated by the short line positioned above each vector DNA. (**a**) An approach used for transgenics produced using *Agrobacterium*, in which a restriction enzyme is used that cuts at a known site within the T-DNA (which may or may not be near the genes of interest) and at an unknown site in the plant DNA. The probe is typically located very near or at the right border (RB). (**b**) An approach used for transgenics produced via direct DNA delivery, in which a restriction enzyme that does not cut within the vector DNA is used. Because the vector–plant DNA junctions are not as well defined as for transformants produced using *Agrobacterium*, it is, typically, useful to employ a transgene coding sequence (particularly the selectable marker gene) as the probe as these sequences are the least likely to have been disrupted during integration. (**c**) An alternative approach, in which a restriction enzyme is used that cuts once within the vector DNA. In this approach it is useful to use an enzyme that does not cut within (or immediately flanking) a PTU and, as for approach (b), employing a probe from a PTU coding sequence is recommended.

Concluding comments

As noted earlier, analysis of transformed plant material has one of two objectives: determining if the material is transgenic and its characterization. Various molecular and biochemical techniques are available for such analysis but, as described in this review, each addresses the objectives differently. Therefore, quality analytical data must always be considered the product of not only technical competence but also an understanding of both the experimental objectives and the characteristics of the material being analyzed. A deficiency in any one of these three areas may result in untenable conclusions.

Acknowledgements

I thank David Hondred and Penny Hunst for reviewing the manuscript, and present and past colleagues at Pioneer Hi-Bred and Garst/Zeneca Seeds for helping shape the thoughts and approaches presented here.

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Counting and classifying possible protein folds

Adam Godzik

Proteins come with a bewildering variety of sequences, structures and functions. Understanding the relations between these three aspects is one of the main unsolved problems of modern day biology. But before significant progress can be made, we have to make sense of the apparently chaotic sea of known protein structures. In the last few years, thanks to the newly developed techniques of structure analysis, a dramatic increase in the number of known protein structures has been achieved. It is now known that protein structures are organized around several common themes. Classification of proteins into structural families, which is a subject of this review, is built on the discovery and analysis of such regularities and similarities.

The first known protein structure was described as pursuing 'a complicated course, turning through large angles and generally behaving so irregularly that it is difficult to describe the arrangement in simple terms'[1]. Someone viewing a protein structure for the first time would probably agree. Almost 40 years since this initial study, over 3000 protein structures have been characterized[2], but we are still unable to predict protein structures from amino acid sequences, making the 'protein folding' problem one of the most persistent questions in molecular biology. However, we have discovered many regularities in these apparently chaotic structures, and used them to classify the universe of known protein structures into families and neighborhoods.

Such classifications are much more than just organizing the unknown. Two simple observations demonstrate that the classification schemes form an integral part of all structure prediction methods. The first observation is that proteins with similar sequences always form similar structures and usually have similar or related functions. Even for the recently discovered organisms from the *archea* domain up to 30–40% of the complete genome can be recognized as encoding products that belong to one of the known protein families[3]. Homology modeling of new structures is now a standard tool in many applications, such as structure-based drug design[4]. The second observation is that many proteins share some similarity in their three-dimensional structure, despite the lack of any recognizable similarity between their sequences. The reasons for the existence of such 'structural twins' are unknown but this observation has been used in a new class of structure prediction methods. In the threading approach[5–10], a sequence is repeatedly 'threaded' through each of many structures from a database of known proteins and a simplified fitness function is used to score each threading. The aim is to find a 'least worst' structure for a given sequence, and thus to recognize a possible 'structural twin' for a new protein.

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Levels of protein structure

Proteins are linear heteropolymers built from 20 common amino acids. Ignoring the side groups, the polypeptide backbone forms a line tracing a complicated path through space. The comparison of two such paths is far from trivial, and recognizing and quantifying a similarity between two paths remains a problem. Methods ranging from knot theory to various algorithms adopted from the graph theory, computer vision and several other fields have been employed. A similarity between two protein chains, once identified and expressed in the form of an alignment, can be quantified by various structure similarity measures. The root mean square of the distance (RMSD) after optimal superposition is the most common similarity measure, but other measures, such as distance matrix difference, contact map overlap or similarity between structural neighborhoods, have also been used. Recently, it was shown that the different measures of similarity between protein structures used in various structure alignment algorithms result in subtle, but significant, differences in how the positions of two structures are equivalenced[11,12]. However, general

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PII: S0167-7799(97)01030-5

Glossary

Protein architecture – A particular packing of secondary structure elements, disregarding an order of connection between them.
Protein topology – A particular connection of secondary structure elements, a specific example of protein architecture.
SSE – Secondary Structure Element. Fragment of a protein chain with regular local structure.

structural features, such as those discussed here, do not depend critically on details of structural comparison.

In contrast to the global shape of the protein chain, the local conformation of the protein is often fairly regular, with about 50% of the total length of all known proteins in one of two conformations: helical or extended. Such locally regular fragments, commonly referred to as secondary structure elements (SSEs; see Glossary), have a mean length of about ten (α-helix) or six (β-strand) residues. Thus, a protein topology can be described in terms of SSEs and their mutual orientation. Compare, for instance, the flavodoxin molecule, as shown in an identical view as a backbone trace in Fig. 1a and as a collection of SSEs in Fig. 1b.

With this description, some regularities, as well as similarities between various protein structures, become immediately obvious. For instance, β strands linked to each other by a network of hydrogen bonds form twisted sheets. The structure of several protein families can be described as being built from two parallel β sheets (see Figs 2a and 2d). There are many possible topologies within this general architecture[13], as shown for the B domain of this histocompatibility complex I (Figs 2b and 2e) and the first domain γ-crystallin (Figs 2c and 2f).

The apparently chaotic protein chain often shows a surprising level of symmetry when represented in terms of SSEs. This representation, occasionally used from the late 1950s onwards by crystallographers discussing structures of newly solved proteins, was elevated to the status of a paradigm in protein structure studies by J. Richardson in the early 1980s[14]. The strange beauty of protein structures when shown by the ribbon diagrams (e.g. Fig. 1b), definitely contributed to an immediate acceptance of this new way of thinking about proteins.

Protein structure classification schemes

With the new picture of protein structure it was easier to notice similarities between different proteins and thus protein structure classification schemes were born. Several groups have attempted a complete classification of all known protein structures, and most of these classifications are available over the Internet[15–18]. A typical protein structure classification looks like a tree, with branches dividing at every level into 'thinner' branches. On the first level, proteins are usually divided according to the dominant secondary structure into all-α, all-β and mixed, which in turn can be divided into the α/β group, where α-helices and β-strands alternate along the chain, and the α+β group, where they form separate sub-domains within the protein. On the next level, typical arrangements of blocks of secondary structure elements for every structure class are listed. For instance, the CATH classification scheme developed at the University of London[16] classifies all-β proteins into barrels, sandwiches, propellers and trefoils, depending on the overall number and organization of β sheets. The actual arrangement of individual SSEs within each of the broader classes defines the next level of the tree. For instance, the immunoglobulin domain (Fig. 2) would be classified as an all-β (first branch), two-layered sandwich (second branch) with a Greek key topology (third branch). The SCOP classification developed at the MRC in Cambridge[15] uses a smaller number of levels. For instance, the all-β proteins are immediately divided into over 50 groups, where Greek key β sandwiches form over five independent structural families, depending on subtle differences in packing between individual strands as well as functional differences between protein groups.

Other types of classification schemes are based on a straightforward comparison of all known protein structures, which are later clustered according to the similarity score. For instance, Holm and Sander[18] used the weighted difference of distance matrices between all pairs of proteins to construct the topology tree. Different levels of the tree are defined by the similarity between proteins, as measured by the standard deviation of similarities between randomly chosen proteins. An analogous all-against-all comparison and subsequent clustering of similar structures has been used in conjunction with several other similarity measures[19–21].

All existing classifications are similar to each other. There are occasional differences caused by weakly similar structures, which might be classified in the same group according to a more robust measure but form two independent groups according to some other measures. Based on such classifications, the estimate for the total number of protein structural families discovered so far is 200–300.

Global analysis of protein structures

Analysis of any of the classification schemes reinforces the observation, discussed earlier, that many structural groups have members from several apparently unrelated protein families. In such cases we are left with several possible explanations: (1) that there is an evolutionary relationship, but it is so distant that it is impossible to detect; (2) that convergent evolution has occurred as a result of common structure/function requirements; or (3) that only a limited number of protein topologies is possible, i.e. different protein families independently utilize the same topology from a small list of choices[22].

Detailed structure comparisons are often able to clarify and sometimes settle questions of evolutionary

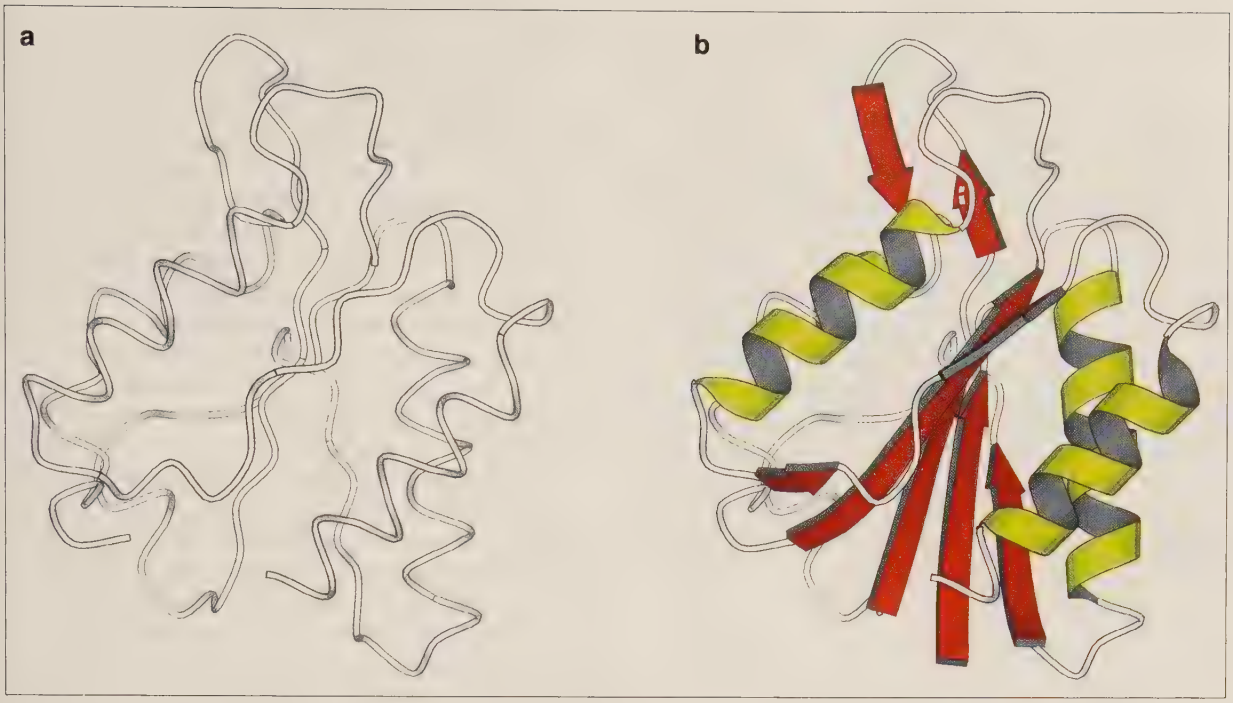

Figure 1
The structure of flavodoxin, as represented by its Cα trace (**a**) and by a ribbon diagram (**b**). Both pictures were prepared with the MOLSCRIPT program[38].

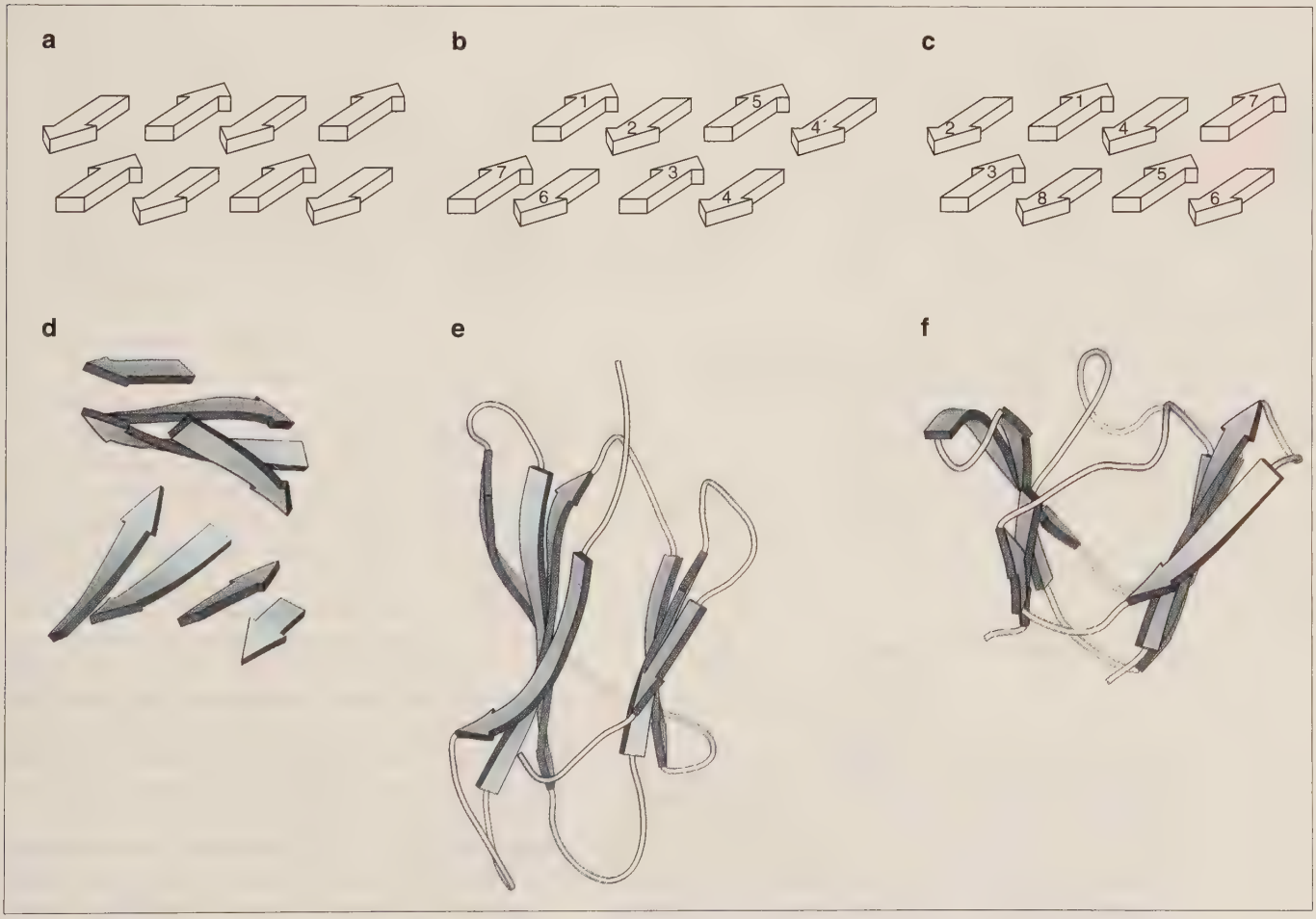

Figure 2
The idealized β sandwich (**a**) and two possible topologies within this architecture, as realized by the B domain of the histocompatibility complex I (**b**) and the first domain of γ-crystallin (**c**). The actual realization of the sandwich architecture is shown in (**d**) and of both topologies in (**e**) and (**f**).

relationships between proteins with similar structures[23,24]. In several cases, structural similarity measures were used to discover distant homology between 'structural twins' that subsequently was confirmed at the sequence and functional level[25].

A choice between the above three possibilities is fundamental because it not only influences our thinking about the protein sequence–structure–function relationship, but also indicates the most efficient structure prediction strategies. For instance, if the existence of 'structural twins' can be explained by extremely distant homologies, this would argue for increasing the level of sophistication of the sequence alignment tools. Mechanism number two would argue for further development of threading methods and number three for the development of topology enumeration methods. Of course different explanations might have to be invoked in different cases and, probably, all three strategies need to be developed further.

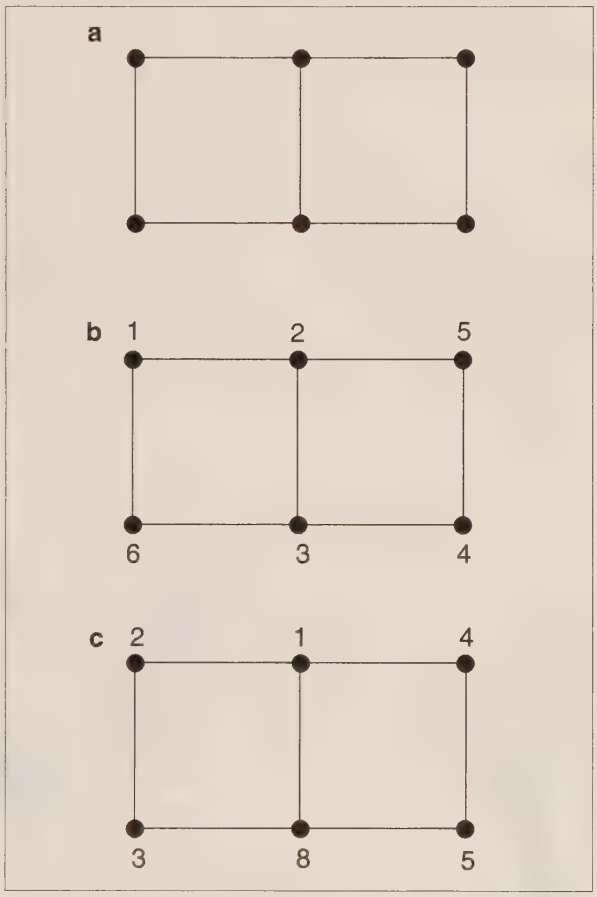

Figure 3

Further idealization of protein topologies in the language of graph theory. (**a**) Unlabeled graph representing an idealized β-sandwich. This graph is analogous to Figure 2(a), but now the information about directions of beta strands is lost. Only three β-strands in each sheet are shown, to emphasize the similarity between the two proteins. Labeled graphs describing the topology of the B domain of the histocompatibility complex (**b**) and the first domain of γ-crystallin (**c**). Note that graph edges describe the interactions between the secondary structure elements, and not connections between them (see text).

How many protein topologies?

The most straightforward estimate of the number of possible protein folds was made by a simple extrapolation of the pace of discovery of new protein folds, disregarding possible mechanisms that limit the number of folds[26]. According to this estimate, the number of possible protein folds is in the order of 1000, with about 10% already known. It was suggested that this estimate may be too low because it does not take into account that some protein topologies are much more widespread than others. Correcting for this effect would increase the estimate to about 7000 (Ref. 27). With both these estimates, there is no way of predicting what the protein folds that have yet to be discovered, might look like.

Less clear are the conclusions reached by estimating the number of possible self-avoiding paths, as distinguished by a threshold difference in the root mean square deviation between different paths[28]. The interpretation of this estimate in terms of protein topologies is not straightforward, as it is not clear if all different self-avoiding paths could be realized as protein chains. A second problem is that it is not obvious what similarity threshold should be used to differentiate between protein folds. Estimates for the number of possible folds depend critically on the latter question. For instance, using an extremely vague similarity measure, which would not always differentiate between a protein and its mirror image, Crippen and Maiorov[28] arrived at an estimated 128 possible chain topologies for chains no longer than 170 residues, with 100 of them identified among known protein structures. However, using a more realistic similarity threshold value dramatically increased the number of possible protein topologies to about 10^{28} for the same chain length. An important aspect of this approach is that it does not depend on any discrete classification scheme.

Other attempts at enumeration of possible protein topologies have concentrated on specific groups of proteins. For all-α proteins, enumeration of all possible topologies consistent with the known rules of protein folding yielded an estimate of 1264 possible topologies for proteins built with up to six helices[29]. Two other protein architectures used in enumerations following similar lines were α+β proteins built from two helices and three strands[30] and β proteins built from two layers of four strands each[13]. In both cases, explicit, even if highly idealized models of new (as yet unseen in nature) proteins could be built. For both β and α proteins, enumeration of protein topologies is based on the idea of a stack: the description of the mutual positions of α and β segments in space, ignoring their direction and position along the chain[22]. Actual topologies can be created from the stack by enumerating various permutations of the secondary structure in the stack. This idea is very close to the idea of protein architecture discussed earlier and to the notion of common spatial arrangements of backbone fragments (SARFs)[27]. Alexandrov and Go[27] identified 50 SARFs, which account for the over 380 unique

protein topologies they have identified in the current release of PDB[2]. It is easy to imagine that enumerating all possible permutations of secondary structure elements in the known SARFs, following the rules set by Finkelstein and co-workers[22], would provide a lower bound estimate to the number of possible protein topologies.

Are there any stacks, SARFs or architectures (depending on which nomenclature is used) still not known among protein structures discovered so far? The CATH classification lists several empty architectures but so far there has been no systematic approach to this problem.

The answer could be provided by yet another approach to classification of protein topologies, based on the analogy between a protein structure and a graph[31–33]. In this analogy, SSEs are equivalent to vertices of a graph and the interactions between segments to edges of a graph. A protein topology is represented by a labeled graph with consecutive numbers of segments as vertices labels and a protein architecture by an unlabeled graph (Fig. 3). Enumeration theorems of graph theory[34] could be used to enumerate all existing labeled and unlabeled graphs with a given number of vertices (i.e. segments) and edges (i.e. interaction regions)[35]. A complete enumeration for a graph with eight vertices and ten edges lists over 700 000 different graphs divided into 236 significantly different (i.e. non-isomorphic) arrangements of linkers. The total number of graphs having up to ten vertices is well over 10^7. The number of possible protein topologies is obviously much smaller. For instance, while we can build a graph for every protein topology, the reverse is not always true. There are also many empirical rules followed by protein topologies, which exclude many possible graphs. Currently, the best upper estimate for the number of possible protein topologies for proteins built from up to 12 SSEs, based on the graph enumeration approach, stands at around 10^4 (Ref. 35).

Conclusions

Protein structure classifications have very interesting and far-reaching applications. Many protein features (such as internal repeats or symmetries), regularities in active site positions with respect to major structural elements, and subtle similarities between proteins become visible only when identified across a broad structural class. When combined with the complete enumeration of possible protein topologies, classification schemes could be used to enhance protein structure predictions. For instance, conformational searches in *ab initio* folding[36] simulations could be performed by complete enumeration of possible topologies. For smaller numbers of linkers and/or by using additional information available from experiments it might be possible to limit the search to 100–200 conformations. Threading predictions could be based on the complete library of all possible topologies, thus removing the principal limitations of this technique. Threading with idealized folds, such as those constructed from enumeration techniques described here, has been shown to be possible, albeit on a very limited number of examples[13,37].

Acknowledgements

I would like to thank Dr A. Finkelstein for many helpful suggestions. This research was supported by the NSF grant MCB-9506278.

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Library snapshots

Molecular Diversity and Combinatorial Chemistry: Libraries and Drug Discovery

edited by Irwin M. Chaiken and Kim D. Janda, American Chemical Society, 1996. US$109.95 (ix + 328 pages) ISBN 0 8412 3450 7

In its Symposium series, the American Chemical Society aims to publish books that are 'snapshots in time' of the area of research they cover, as stated in the foreword. With its latest publication in this series, *Molecular Diversity and Combinatorial Chemistry*, it has done that very successfully.

The book is a compilation of papers presented at two meetings on combinatorial chemistry and libraries organized by the Cambridge Healthtech Institute early in 1996. It contains 27 papers, which have been grouped into six sections: strategies, library design, biology-based chemical libraries, automated solid-phase synthesis, analytical methods and screening, and applications.

The first section, on strategy, is small and a bit disappointing because no novel concepts are discussed. However, the first paper in this section, on the automated synthesis of reverse turn peptidomimetics, contains some interesting new data. Observing that many peptide ligands adopt a reverse turn conformation when interacting with G-protein-coupled receptors, Michael Kahn and co-workers synthesized a 4000-compound library of conformationally restrained reverse turn mimetics and screened it against ten G-protein-coupled receptors. They report hits (IC_{50} = <1 μM) for five of the ten receptors tested with this library. The other two papers in this section cover construct diversity as an alternative to the template and oligomer approaches to libraries, and the need for integrating structure-based design and automated synthesis.

About one-third of the book is dedicated to library design, and those interested more in practical applications than an overview of current research in the field will find some interesting papers here. The majority of papers in this section describe the synthesis of new scaffolds/templates by either solid- or solution-phase methods. None of the papers contain the exact experimental procedures of the reactions described but the information given in the text and the synthetic schemes seems to be very comprehensive. One paper in this part of the book deals with a more unusual type of library; it describes the design and screening of a library of transition metal complexes as potential catalysts for C–H-insertion and cyclopropanation reactions. Screens for catalyst activity are only now starting to emerge, and no doubt we will hear a lot more of this library application in future.

The much smaller section on biology-based chemical libraries contains a very good paper on the use of enzymes for library construction that includes examples of a wide range of possible enzymatic conversions. Another excellent paper in this section discusses the potential of gene transfer for generating 'living' libraries.

For those involved in buying automated-synthesis technology, the 'automated solid-phase synthesis' section may be of interest. Representatives from Bohdan, Argonaut, Diversomer Technologies (Parke-Davis), Alanex and MDL Information Systems survey some workstations and software currently available for automated synthesis.

The short analytical section reviews new IR and NMR techniques for the characterization of compounds on solid-phase beads, and also contains a report of a new cell-based high-throughput assay for G-protein-coupled receptors.

Of course a book on libraries would not be complete without a section on diversity. New software for assessing the diversity of a library is discussed in the final section of the book.

Molecular Diversity and Combinatorial Chemistry gives a very comprehensive overview of recent developments in the combinatorial chemistry field and is recommended to anyone who takes an interest in this area.

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Linking ethical dilemmas

Promise and Perils of Biotechnology: Genetic Testing

University of California, Cold Spring Harbor Laboratory Press and Pyramid Media, 1996. US$70.00 (video + 33 pages) ISBN 0 87969 493 9; VHS 0 87969 494 7 PAL

Promise and Perils of Biotechnology: Genetic Testing is a video (running time approx. 25 minutes) with a 33-page accompanying Teacher's Guide. I viewed the video with a class of eighteen first year biosciences undergraduate volunteers. Following the film we discussed their reaction to it; more of this later.

The video has the following objectives:

- 'give a basic understanding of genetics and genetic testing;
- introduce the Human Genome Project;
- show the difference between inheriting a genetic disorder and inheriting a predisposition to a disorder;
- convey some of the ethical and societal issues that result from the use of biotechnology;
- stimulate discussion about how the information generated by genetic profiles can affect a person's life, career, and society as a whole.'

The aims are largely met, although superficially in the case of the first objective. The explanation of the tests carried out is sketchy but there is a good representation of the difference in scale between the human genome and a single gene.

The film devotes most of its running time to two contrasting cases: Jennifer, tested for Huntington's Disease ('Jennifer knows that if she tests positive for the disease there is no cure. She then risks passing the gene on to her children, should she decide to have any.') and Lily and Laura Scholer who have been tested positive for familial hypercholesterolaemia ('...the Scholers have the opportunity to reduce their risk of heart attack by changing their lifestyles. However, they still grapple with how the knowledge of their condition will affect their ability to get certain jobs and insurance.').

The video demonstrates well how scientific information can be combined with other knowledge and personal values. It explains the purpose of the human genome project and gives a reasonable representation of genetic predisposition to disease, although it is unfortunate that the dialogue near the beginning of the film includes the abbreviation 'PKU' without explanation. My overall impression of the film was that it viewed very much like a television documentary (in the UK rather like the programme QED). This makes it interesting to watch but do not expect much in the way of hard science. There are some tense moments: the examples and families interviewed are genuine, you cannot rely on a happy ending. Indeed it may be difficult to show the video to groups of students if any members of the audience have family members who suffer from an inherited disease.

My student audience found the film interesting and understandable. None found it a waste of time but similarly none thought it essential viewing. On balance the film was considered well-suited to schools and non-specialist audiences. Certainly it would make excellent viewing for social scientists, undergraduate nurses, etc. My students thought the film was a little sketchy on the science for undergraduate biologists but they agreed that it would be wrong for biologists to graduate without ever being exposed to the dilemmas presented by modern technology.

The accompanying book is a guide for teachers, which creates a framework for basing class discussion around the film. It provides six activities, templates for handouts, scenarios and summaries on 15 inherited disorders (fortunately including PKU, so if you read the book you are prepared for the inevitable question). There is a useful reference section, which is annotated with advice on the content and suitability of readership. There is a brief glossary with one glaring ommission, PCR; a test alluded to in the video but never named.

In summary the package is interesting, relevant, provocative and interactive. It is well suited to advanced pre-university science education, including foundation year or access programmes. It is relevant to specialist biochemistry and genetics first year undergraduates but the package is not designed for this specific audience. It is worth the money and I feel the video and accompanying exercises and discussion are guaranteed to stimulate interest and encourage student participation.

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Nucleic acids update

Nucleic Acids in Chemistry and Biology (2nd edn)

edited by G. M. Blackburn and M. J. Gait, Oxford University Press, 1996. UK£29.95 (xix + 528 pages) ISBN 0 19 963533 1

This is the second edition of the successful text *Nucleic Acids in Chemistry and Biology* published in 1990. The text consists of ten chapters and a helpful 13 page glossary. Following an introductory scene-setting chapter by the editors, the rest of the contributions are sensibly divided into chapters concerned with different aspects of the chemistry and biology of nucleic acids.

Chapter 2 discusses the structure of both RNA and DNA and, in the light of new research, includes a new, detailed and more accurate account of tertiary structure than that given in the first edition. Chapter 3 deals with the chemical synthesis of nucleic acids, with the sections on the synthesis of oligonucleotide analogues and oligoribonucleotides having been completely revised in this edition. Chapter 4, which deals with the biosynthesis of nucleic acids, ends with a discussion of the use of new drugs for the inhibition of nucleic acid biosynthesis as applied to anticancer and antiviral therapy. Chapter 5 discusses structure, replication and rearrangements in prokaryotic and eukaryotic genomes with more emphasis on DNA recombination. Chapter 6 is on RNA sequences and their transmission; this edition includes a more extensive discussion of catalytic RNA as well as new ideas regarding folding and tertiary interactions of RNA molecules. The covalent and the reversible interactions of nucleic acids with various small molecules (including a good account of the effects of the potent antitumour enediyne antibiotics) are considered in Chapters 7 and 8, respectively. Chapter 9 has been updated and includes a very good account of the current knowledge of the interactions of proteins with nucleic acids. The final chapter discusses the concepts behind some of the main techniques used for the study of the nucleic acids.

Each chapter contains a useful summary and an extensive list of pertinent references. There are many good two-tone illustrations throughout, with Chapter 9 including full colour photographs from X-ray crystallographic studies.

Generally speaking, Mike Blackburn and Mike Gait have done an excellent job of integrating the work of nine different contributors with their own to produce a book that is well written, easy to follow and very instructive. This up-to-date book should be on the bookshelf in the studies of both students and teachers as well as of active research workers and it should be available in the biochemistry section of every library at institutions with relevant teaching and research interests.

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Letter

Next-generation Fc chimeric proteins: avoiding immune-system interactions

Ersatz chimeric products of therapeutic proteins have been prepared with Fc domains to confer on these proteins the prolonged *in vivo* survival of IgG. However, their introduction into clinical settings may have unintended adverse consequences because of continued potential to interact with immune-system components. One recent example is instructive. Patients treated with a tumor-necrosis-factor-receptor–IgG-Fc (TNFR:Fc) chimera to suppress the systemic effects of TNF_{α} production during sepsis experienced significantly higher mortality[1]. No cause for the excess deaths was established other than the suggestion that TNF may be essential during sepsis.

However, an alternative explanation may instead apply that is directly pertinent to our thesis. That is, TNFR:Fc may mediate attack against the producers of TNF (i.e. activated macrophages, monocytes and other cells that are also key components of the antibacterial systemic response[2]). TNF is expressed as a transmembrane protein that is proteolytically cleaved to release the mature soluble molecule, and a significant membrane representation is retained on producer cells[2]. An outcome that targets these producer cells could be harmful to the host in its attempt to fight bacterial infection, quite apart from whether TNF itself has any role in that response. In the absence of infection, compromise of the few cells activated to express TNF would not reveal itself clinically, and the cited tolerance of TNFR:Fc in uninfected subjects[1] is not surprising.

Via the Fc_{γ} receptors (FcR)-I, -II and -III, all isotypes of human IgG Fc can potentially direct granulocytes, monocytes/macrophages and/or natural killer cells to attack coated targets by antibody-dependent cellular cytotoxicity (ADCC) and/or phagocytosis[3]. These FcRs may also induce death of coated cells by apoptosis[4] or alternatively promote cellular activation[5] by fostering multivalent clustering of cellular ligands in settings where simple uni- or bivalent interactions would not induce such effects[4,5]. Further, all isotypes of IgG Fc, stripped of their Fab arms, can fix complement[6] and may continue to do so in chimeric configurations with other proteins. Accordingly, the selection of different human IgG isotypes for therapeutic use in humans may not escape unwanted immune-system interactions.

Instead, the next generation of Fc chimeras will need to have the domains that interact with hematopoietic FcR (Ref. 7) and complement[8] mutated out, without disturbing the distinct structural regions of the Fc_{γ} that interact with the vascular endothelial FcRB (Brambell receptor; Ref. 9) to confer long survivals *in vivo*. Such re-engineered IgG-derived molecules may provide a safer route for human clinical applications by avoiding the aforementioned concerns and their potentially lethal consequences. Although the proposed scenario for toxicity of TNFR:Fc is speculative, it will be difficult to disprove. Thus, these hazards will remain a potential risk for future applications unless such deleterious immune-system interactions can be absolutely excluded.

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Meeting report

Phage display and antibody engineering: a French overview

New versions of antibodies that exhibit improved binding properties, reduced immunogenicity and novel effector functions can be obtained by various molecular engineering approaches, including phage display. This technique, initially set up for the identification and manipulation of peptides[1], is now used as a complement to hybridoma technology and represents a powerful tool for the selection and cloning of antibody fragments. Phage display on the surface of filamentous phages was first introduced in the mid-1980s as a means of screening libraries of peptides, or of studying protein–protein interactions. One of its most spectacular developments has been its extension to the display of antibody fragments, either as Fabs or as single-chain Fvs (scFvs)[2]. Rare phages encoding antigen-binding activities can be selected by screening libraries by using rounds of adsorption–elution on surfaces bearing antigens or on antigen-positive cells. The technology has now been appropriated by a growing number of research groups from around the world, each with a major interest in generating useful reagents of human origin for clinical purposes. A recent meeting*, at which most of the participants were French scientists, provided an opportunity to discuss the progress and difficulties in developing phage-display technology, antibody engineering and molecular targeting.

*The meeting 'Banques d'Expression à la Surface de Phages et Vectorisation/Phage Display Libraries and Recombinant Ab-mediated Targeting' was supported by the Fondation Mérieux, Pasteur Mérieux Connaught and Pharmacia Biotech SA, and was held in Annecy, France, 28–30 October 1996.

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Combinatorial libraries: generation, screening and products

Strategies for making large antibody repertoires in phages to isolate rare antibody fragments with useful specificities and high affinities have been explored. Combinatorial infections allow a large repertoire of antibodies to be generated by first transforming *Escherichia coli* with a repertoire of heavy chains encoded on plasmids and subsequent infection with a repertoire of light chains encoded on phages (or vice versa). The heavy- and light-chain genes are then locked together within the same phage particle using the *lox*–Cre site-specific recombination system of bacteriophage P1 (Ref. 3). Frédérique Geoffroy (Pasteur Mérieux Connaught, Marcy l'Etoile, France) described another recombination system, this time involving the λ phage site-specific *att*–Int integration system; this can also lead to the irreversible physical association of V_H and V_L sequences. Further improvements to this approach, including the use of new vectors (shuffling of replication origins, sequence exchange between plasmid and phage rather than sequence fusion), have overcome the previous drawback of the presence of the parental phagemid even after recombination and allow the easy generation of libraries of up to 10^{10} clones.

It is also possible to generate large phage-display libraries by strategies based on the isolation of the Ig repertoire of several donors, either immunized or not (the latter leading to 'naive' libraries), by cloning pooled V_H and V_L fragments. To obtain such libraries, PCR amplimers corresponding to all known variable gene families are used. In addition, the use of B cells from different sources (peripheral blood, spleen, lymph node, bone marrow), as well as their *in vitro* activation, allows the size of the repertoire selected to be increased (Régis Sodoyer; Pasteur Mérieux Connaught). However, the possibility that phage-display libraries may contain many polyspecific antibody fragments, specifically when derived from naive donors, was pointed out by Stratis Avrameas (Institut Pasteur, Paris, France). Analysis of human or animal sera showed the presence of IgM, IgG and IgA antibodies that bind to a large number of antigens. These antibodies, mostly encoded by germ-line genes with a few point mutations, can bind both endogenous and exogenous antigens. They can block or facilitate cell growth or infection, and may be involved in the development or the control of autoimmune diseases. Thus, the specificity of antibody fragments isolated from phage-display libraries, in particular those derived from naive donors, should be carefully evaluated before their use in clinical trials. Fully synthetic libraries, as discussed by Andreas Pluckthün (Institute of Biochemistry, Zurich, Switzerland), in which all complementary-determining regions (CDRs) are randomized on given frameworks could be considered as an alternative to naive libraries. The in-cell PCR technique (a PCR performed in fixed and permeabilized cells) allows the assembly of the original V_H–V_L pair expressed within single cells. This can be an elegant way to study the Ig repertoire present in organs heavily infiltrated by B cells during autoimmune diseases, as well as enabling the isolation of autoantibody fragments (Nicolas Chapal; CNRS, Montpellier, France).

Nevertheless, phage-display libraries allow the selection of antibody fragments with exquisite specificity. For instance, the combined use of molecular modeling and phage display of scFvs directed to cortisol and steroid analogues, followed by recursive PCR mutagenesis, allowed Patrick Chames (CNRS, Marseille, France) to isolate variant scFvs exhibiting more restricted or new specificities. Similarly, two scFvs against crotoxin (a South American snake toxin) isolated from a human semisynthetic library[4] exhibited different biological activities, with one of them triggering acetylcholine release and dissociation of the two CA and CB crotoxin subunits (Pierre Lafaye; Institut Pasteur).

Phage display of scFvs derived from established hybridomas is also very useful in analyzing antigen–antibody interactions. Frédéric Ducancel (DSV-CEA, Saclay, France) reported the functional mapping of the paratope of an anti-sea-snake-toxin antibody that partially mimics the nicotinic acetylcholine receptor. Patrick England (Institut Pasteur) presented an elegant study on anti-lysozyme D1.3 and hen-egg lysozyme (HEL) interactions. Site-directed mutagenesis of defined CDR3 V_H and V_L amino acid residues showed the role of both aromatic and non-aromatic residues in the D1.3–HEL interface, and the energetic importance of hydrogen bonds due to water molecules present between D1.3 and HEL. A detailed analysis of an scFv against the CA crotoxin subunit allowed Valérie Choumet (Institut Pasteur) to demonstrate a strong homology between two regions of the CB crotoxin subunit directly involved in CA subunit binding and CDR2 and CDR3 of the scFv; this suggests molecular mimicry between the CB subunit and the scFv paratope.

Conversely, antibodies can be used to select mimotopes from phage display peptide libraries, particularly for use as vaccine antigens (Monique Vogel; Hôpital de l'Ile, Berne, Switzerland). Of note was the observation that the use of cyclic peptides (constrained by a disulfide bond) made it possible to isolate clones from a small number of selection rounds (usually three) and that peptide-displaying phages could be used directly as immunogens (Caroline Demangel; Institut Pasteur). Phages can also be engineered to express proteins such as p53 (Luc Aujame; Pasteur Mérieux Connaught) or the epidermal growth factor (EGF) (Christelle Souriau; CNRS, Montpellier) as fusion proteins with g3p or g6p, making them valuable tools for analyzing the biological activity of such molecules.

The elegant and potentially powerful approach of co-selecting both antibody and antigen specificity was also described by Andreas Pluckthün. In the selective-infective phage (SIP) method, an antigen is fused to the N-terminal region of g3p and antibody is fused to the C-terminal region: infectivity of phage particles can be restored only upon the proper association of cognate pairs.

A number of other reports were concerned with the isolation of specific antibody fragments. Phage-display libraries have been used to select scFvs directed to dsDNA or to nucleosomes (Fabien Jovelin; Hôpital Charles Nicolle, Rouen, France). Véronique Barban (Pasteur Mérieux Connaught) discussed her efforts to isolate anti-hepatitis-C virus (HCV) Fabs from a library made from samples taken from recovering patients, while Jean-Luc Chagnaud (INSERM, Bordeaux) presented his progress towards the isolation of anti-human-T-cell-leukemia-virus-I (HTLV-I) scFvs. Anti-rhesus-D (RhD) antibodies isolated from a library derived from a hyperimmunized individual were also discussed (Sylvie Miescher; Hôpital de l'Ile). Efforts towards the isolation of anti-*E. coli* lipopolysaccharide were also described and it was shown that such antibodies could indeed be presented at the surface of phages (Catherine

Manin; Pasteur Mérieux Connaught). Also of interest was the characterization of catalytic antibodies (DNases or proteases) derived from patients with autoimmune diseases (Alain Friboulet; CNRS, Compiègne, France). However, both the functional role of and the clinical interest in these recombinant antibody fragments, such as the scFv that neutralizes rabies virus (Bruno Muller; Institut Pasteur), remain to be clearly established.

Phage-display-derived antibody fragments and recombinant antibodies: towards clinical use?

Some Fabs or scFvs derived from phage libraries are potentially useful therapeutic molecules for a variety of pathological conditions in humans. Although their *in vivo* use is still in its early stages, several recombinant antibody fragments are currently being evaluated *in vitro* and in animal models. A major difficulty lies in the low yield of these molecules when produced in *E. coli*. Their production in insect cells, after cloning of Fabs or scFvs in baculovirus or following expression in B cell lines of reconstructed antibodies of various isotypes, represents an alternative to production in *E. coli* that results in enough material for evaluations of their role *in vivo*. For example, Corinne Lemeule (CNRS, Montpellier) demonstrated the expression in insect cells of an scFv against digoxin, a cardioactive glycoside used in the treatment of heart failure, and Philippe Klein (Laboratoire Français du Fractionnement et des Biotechnologies, Lille, France) discussed the production of human anti-RhD recombinant antibodies, with variable regions joined to different constant regions, for the prevention of RhD allo-immunization. Alternatively, the *in vivo* transfer of genes encoding a recombinant virus-neutralizing antibody (as anti-human-immunodeficiency-virus-1) or an immunotoxin, which results in production of the desired protein *in situ*, can overcome *in vitro* production difficulties (Majid Mehtali; Transgène SA, Strasbourg, France).

The possible use of engineered recombinant antibody fragments as non-viral transfer vectors was discussed by Christophe Demaison (Institut Pasteur), who reported the generation of a fusion molecule containing anti-dsDNA Fab and interleukin 2 (IL-2) to target plasmid DNA into cells expressing IL-2 receptors (IL-2R positive cells). Moreover, IL-2 receptor targeting with antitumor scFv–IL-2 fusion molecules could be an interesting alternative to the use of high doses of IL-2, which provoke severe side-effects in cancer patients. The increase in the concentration of IL-2 in the tumor environment realized through the action of scFv could lower the dose of IL-2 required to get a beneficial therapeutic effect. An scFv against disialoganglioside (G_{D2}) was fused to IL-2 and subsequently showed excellent binding to both IL-2R-positive T or natural killer cells and to G_{D2}-positive neuroblastoma cancer cells; in addition, it triggered the proliferation and cytotoxicity of lymphokine-activated killer cells (Jean Michon; Institut Curie, Paris, France).

Recombinant antibody fragments can also be used to redirect retroviral infection. The display of scFvs against major histocompatibility class I molecules, of EGF or of a binding domain for the Ram-1 phosphate transporter at the N-terminus of MoMLV SU (a Moloney murine leukemia virus envelope protein) with different spacers, allowed retargeted binding, even though several factors preventing the transduction of the virus following binding were identified – interdomain spacing, viral entry route and the targeted cell-surface molecule (François-Loïc Cosset; CNRS, Villeurbane, France, in collaboration with Stephen Russell; MRC, Cambridge, UK). The acquisition of new cellular tropisms of both retroviruses and adenoviruses by adding heterologous ligands such as antibody fragments was also presented by Majid Mehtali.

Finally, several reports discussed the use of recombinant antibody fragments for intracellular targeting ('intrabodies'). Christine Giovane (ESBS, Illkirch, France) reported the generation of Fabs directed against the E6 protein derived from human papillomavirus type 16; this protein, in association with the E6–AP molecule, triggers the degradation of p53. When expressed in Cos cells, the Fabs induced a growth arrest, which was partially reversed by the overexpression of recombinant E6. Moreover, targeting recombinant antibody fragments to mutated p53 can restore the p53 DNA binding and/or transactivating activity and makes it possible to assess p53 functions *in vivo*. The detailed characterization was presented of two anti-p53 scFvs that were cloned and expressed either as soluble forms or on phage (Pascale Cohen; University of Dundee, UK. Florence Bourgain; INSERM, Paris, France). Similarly, an scFv against p53 that restored the transactivating property of mutated p53 was fused to a synthetic DNA-binding element to activate killer genes under the control of p53 in vectors containing sequences to which the synthetic element binds. Consequently, this system allows p53 mutated cells to be killed (Laurent Bracco; GMD, Rhône-Poulenc Rorer, Vitry-sur-Seine, France). The oncogenic $p21^{ras}$ protein is also a good target for intrabodies. An scFv derived from a neutralizing anti-$p21^{ras}$ antibody, expressed in *Xenopus laevis* oocytes and in eukaryotic cells, specifically inhibited Ras-mediated signaling events and triggered apoptosis of tumor cells. Efficient transduction of this scFv using an adenoviral-vector-elicited tumor regression of $p21^{ras}$-mutated HCT116 colon carcinoma cells grafted to nude mice (Olivier Cochet; Institut Curie, in collaboration with Bruno Tocqué; GMD, Rhône-Poulenc Rorer). Thus, the intracellular production of antioncogene antibody fragments could represent a powerful approach for cancer treatment.

Conclusion

This meeting, the second of its kind, provided participants with a stimulating and thorough overview of antibody-engineering developments in France. Further interaction between groups carrying out basic research on antibody structure and function, as well as on B cell maturation, and those groups focused on the biotechnological aspects of antibody research should stimulate new developments in antibody engineering in France and elsewhere.

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Bioremediation of marine oil spills

Roger C. Prince

Bioremediation is a potentially important option for dealing with marine oil spills. Oil-degrading microorganisms are indigenous to the world's oceans, but environmental constraints limit their activity and so a bioremediation strategy must be tailored to local conditions. While an oil slick is at sea, its biodegradation is likely to be surface-area limited and can be stimulated by employing dispersants. However, if oil reaches a shoreline, alternative options are needed. The addition of fertilizer nutrients has been successful in stimulating oil biodegradation in oligotrophic conditions, and suggests opportunities for designing alternative strategies for other environments.

Marine oil spills catch the public's attention, generating intense pressure on the parties responsible for the spill, backed by legislation, for a prompt and effective response. Fortunately, such spills are rare, and in most years the total amount of oil released into the sea from catastrophic accidents is approximately the same as that introduced by natural oil seeps[1]. Furthermore, it is far less than that released from municipal sources and general transportation. The oil industry and consumers are working to minimize catastrophic releases, and 1995 was the best year to date, with only 5000 tonnes spilled at sea[2]. Unfortunately, the spill from the *Sea Empress* alone (February 1996 off the southern coast of Wales) exceeded this amount in 1996, and it is unlikely that oil spills can be completely prevented. There is thus a continuing need for environmentally-responsible cost-effective tools for responding to oil spills.

Where possible, physical collection of the spilled oil with skimmers is the first response but this is rarely completely successful. Providentially, most crude oils are inherently biodegradable, with estimates ranging from 70 to 90% for different oils[3]. Stimulating this biodegradation is thus an exciting opportunity for biotechnology, and indeed bioremediation of oil was used on more than 120 km of rocky shorelines following the *Exxon Valdez* spill in Alaska in 1989 (Refs 4,5). What are the prospects for expanding on this success?

Approaches for cleanup of floating oil

It is instructive to consider what happens when oil is released into the marine environment. Since almost all commercoally used oils float, spilled oil spreads on water and increases its surface area. This encourages the evaporation of components containing up to about 12 carbons and the dissolution of those few molecules with an appreciable aqueous solubility. The floating slick also absorbs water, and photochemistry oxidizes some compounds in the slick. If it does not beach, the slick will eventually disperse in the water column. This is a natural phenomenon that is aided by wave energy; indeed, if the oil spill occurs in severe weather the oil may be dispersed into the water column in a matter of hours, as happened to the light crude oil from the *Braer* off the Shetland Islands in January 1993[6].

One major option for stimulating biodegradation of spilled oil is to encourage natural dispersion by adding chemical dispersants to the oil slick[7]. The use of dispersants has always been thought to stimulate the natural process of biodegradation because microbial attack is at the oil–water interface, and dispersion of the oil dramatically increases the area available for microbial colonization. Since the oil is dispersed into such a large volume, other nutrients required for the growth of bacteria, such as nitrogen, are likely to be available in sufficient concentrations. Varadaraj *et al.*[8] have recently shown that the surfactants used in some dispersants, including Exxon's COREXITS® 9527 and 9500, further enhance the biodegradation of dispersed oil by providing a digestible substrate that stimulates the growth that can attack the oil. Dispersants containing these surfactants are thus one way of stimulating the rate of biodegradative removal of oil from the marine environment.

Alternative approaches to dealing with floating oil include dispersant formulations containing nitrogenous nutrients[9] or microbial inocula[10], but these are not currently commercially available. There have also been many proposals to add bacteria to oil slicks, and much talk of using genetic engineering to enhance the

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PII: S0167-7799(97)01033-0

degradative capacity of organisms. One commercial product of non-engineered bacteria was tested following the spill from the *Mega Borg* in the Gulf of Mexico in 1990 but no data were collected to show that the product had any effect[11].

Approaches to the cleanup of shoreline oil

If the spill is near land it is likely that some oil will reach the shoreline. Again, physical collection is the response of first choice. On sandy shores, scraping the oil into trenches and recovering it with vacuum trucks, as done following the *Sea Empress* spill[12], is a typical collection procedure. On rocky shores the usual response is to wash the oil back into the sea and collect it with skimmers: this was the method applied following the *Exxon Valdez* spill[13]. Even when chemical beach cleaners are included in the wash water, washing always leaves some residual oil, and this is an attractive target for bioremediation. Research on this topic has been going on for more than 20 years[3,14,15], particularly in Norway and Canada, and more recently there has been substantial work in Japan, Israel, the USA and the UK[14,15].

Bioremediation played a major role in the cleanup of the *Exxon Valdez* spill[4,5]. As noted above, crude oils are inherently biodegradable, but they contain essentially none of the nitrogen, phosphorus or trace elements essential for microbial growth. Some marine environments, particularly those with agricultural input, provide these nutrients but the Alaskan coastlines are oligotrophic. Bioremediation therefore focused on partially alleviating the nutrient limitation by adding fertilizers. Two fertilizers were used: an oleophilic product, Inipol EAP22®, that was designed to adhere to oil and provide fertilizer at the oil–water interface[16], and a slow release agricultural product, Customblen®, that released nutrients with every tidal immersion[4,5]. The approach was very successful, stimulating the natural rate of biodegradation two to five times with no detectable adverse environmental impacts[4,5].

While there have not yet been any other well-documented large-scale applications of fertilizer bioremediation, there have been several experimental confirmations of the basic soundness of this rather simple intervention (see Refs 3,14,15) and the technology is part of the 'tool-box' for dealing with spills on aerobic beaches. But are there more sophisticated approaches that might yield even more dramatic stimulations of the natural rate of biodegradation?

Many vendors offer bacterial inocula to stimulate biodegradation, and the popular conception is that adding bacteria is the basis of bioremediation. Unfortunately, there are no convincing reports showing that adding bacteria is helpful, but there are many that show no beneficial effect. Perhaps the most startling test was performed by the United States Environmental Protection Agency when testing potential inoculants for stimulating oil biodegradation following the *Exxon Valdez* oil spill[17]. Eight commercial products were tested in small laboratory reactors that allowed substantial degradation of oil by the indigenous microorganisms of Prince William Sound; all eight microbial inocula had a greater stimulatory effect on alkane degradation, at least for the first 11 days, if they were sterilized by autoclaving prior to addition! This suggests that the indigenous organisms readily outcompeted the added products, but that autoclaving the products released some trace nutrient(s) that was able to stimulate the growth of the endogenous organisms. One approach to overcoming this competition has been proposed by Rosenberg *et al.*[18]. They claimed that addition of a urea–formaldehyde fertilizer that adheres to oil and selected oil-degrading organisms able to use this fertilizer as a nitrogen source dramatically stimulated oil degradation on a beach in Israel.

Modern molecular genetics offers the promise that genetic engineering will improve the degrading ability of bacteria that could be applied to oil spills, and indeed the landmark patent for genetically engineered organisms was for an organism with hydrocarbon-degrading abilities[19]. However, little progress has been made in developing robust strains of organisms for this purpose, or in overcoming concerns that such organisms should not be released into the environment. Instead, the majority of current research is aimed at understanding the shoreline environment where biodegradation occurs and discerning ways of stimulating the indigenous microorganisms without adverse environmental impacts. These organisms become the basis of a food web that might be optimized for the most rapid cycling of trace elements, thereby maximizing biodegradation. There is certainly optimism that if we were to understand the fundamental microbial ecology of a spill site then we would be able to develop bioremediation into an even more effective tool for the cleanup of oil spills.

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Crystalline cellulose degradation: new insight into the function of cellobiohydrolases

Tuula T. Teeri

Cellulose is an important industrial raw material and a source of renewable energy. The crystalline structure of cellulose is biodegradable by the combined activities of many different enzymes. Cellobiohydrolases, the key cellulases, have extended tunnel-shaped active sites. Owing to the design of their active-site tunnels, they do not make cuts within cellulose chains but release cellobiose from the chain ends. Cellobiohydrolases may therefore prove to be useful for achieving subtle modifications of fiber properties in paper or textiles without the loss of fiber strength commonly observed with other cellulases.

Cellulose is the most abundant biological polymer on earth. It is primarily found as the major structural component of the cell walls of terrestial plants and marine algae but is also produced by other organisms such as some bacteria and marine animals[1]. In addition to its tremendous ecological importance, cellulose from higher plants has been exploited for centuries in a wide variety of practical applications. In modern-day industrial processes, the majority of commercially produced cellulose is used to manufacture paper, textiles or chemical-grade cellulose[1].

Cellulose can be hydrolyzed to soluble sugars either chemically or enzymatically. In many industrial applications, enzymatic hydrolysis is becoming increasingly desirable owing to the mild processing conditions, high specificity of the reactions and better control of the processes[2]. Most current applications of cellulolytic enzymes are carried out using small amounts of crude enzyme mixtures rather than purified enzymes and do not depend much on high specificity. To develop new products and processes based on enzymatic modification and hydrolysis of cellulosic materials, enzymes with well-understood activities are required.

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Native cellulose has a complex and heterogeneous stucture

The physical structure and morphology of native cellulose are complex and the fine details of its structure have been difficult to determine experimentally (reviewed in Refs 3–5). The chemical composition of cellulose is simple, consisting of D-glucose residues linked by β-1,4-glycosidic bonds to form linear polymeric chains of over 10 000 glucose residues. The individual chains adhere to each other along their lengths by hydrogen bonding and van der Waals forces, and crystallize shortly after biosynthesis. An individual cellulose crystal contains tens of glucan chains in a parallel orientation with their reducing chain ends at one terminus of the crystal and their nonreducing chain ends at the other. Although highly crystalline, the structure of cellulose is not uniform. A large body of physical and chemical evidence indicates that native

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PII: S0167-7799(97)01032-9

cellulose contains both highly crystalline and less-ordered amorphous or paracrystalline regions[3–5]. Although the resolution of the methods currently available is not sufficient for identifying the exact nature of the imperfections present in cellulose, it is assumed that various chain dislocations and ends, distorted surfaces, and twisted or strained regions occur along the microfibrils[3–6]. During the biosynthesis of cellulose there is apparently, no strict coordination ensuring that all chains have identical lengths. Consequently, an individual chain may be involved in more than one crystalline region, resulting in the occurrence of occasional chain ends even within the highly ordered crystalline regions[3,7,8] (Fig. 1). Cellulose crystals in the fibers of higher plants associate with other cell-wall components, such as lignin and hemicellulose, resulting in even more complex morphologies.

General features of cellulolytic enzymes

Efficient solubilization of the heterogeneous and highly insoluble native cellulose requires the presence of many different enzymes. A characteristic feature of most cellulases is a domain structure composed of a catalytic domain linked by an extended linker region to one or more cellulose-binding domains (CBDs) (reviewed in Ref. 11). It is well documented that CBDs are required for efficient hydrolysis of crystalline, but not soluble, substrates, although their exact roles are not yet well understood[12].

All cellulolytic enzymes share the same chemical specificity for β-1,4-glycosidic bonds, which they cleave by a general acid-catalyzed hydrolysis, leading to either inversion or retention of the configuration of the anomeric carbon[13]. In terms of their catalytic reaction mechanisms, cellulases resemble other glycoside hydrolases such as lysozymes and amylases, although subtle differences are also found (reviewed in Refs 13,14). However, important differences are found in the specificities of cellulolytic enzymes towards the more macroscopic properties of the substrate (Fig. 1). Because the natural substrate is often too complicated to be useful for detailed characterization of individual cellulases, a wide range of different model substrates and many different assays have been developed and used to demonstrate key functional differences in enzyme properties (Box 1)[15].

The different modes of action of cellulolytic enzymes on the polymeric substrate are commonly described as endo- and exo- types of attack[29] (Fig. 1). A typical endoglucanase cleaves bonds along the length of the cellulose chains, resulting in a rapid decrease in the degree of polymerization (DP) of the substrate[9,10]. Because the glucan chains can remain associated with the rest of the crystal after a single bond cleavage at the surface, it takes a relatively long time before soluble products are observed after an endo- type of attack. In many practical applications, endoglucanase activity has been found to be detrimental to the strength of the fibers[30].

Exoglucanases (also called cellobiohydrolases) are currently thought to be processive enzymes, initiating their action from the ends of the cellulose chains[10,14,27,28,31–33]. They can attack the crystalline parts of the substrate, producing primarily cellobiose and decreasing the substrate DP only very slowly[10,23]. The complementary activities of endo- and exoglucanases

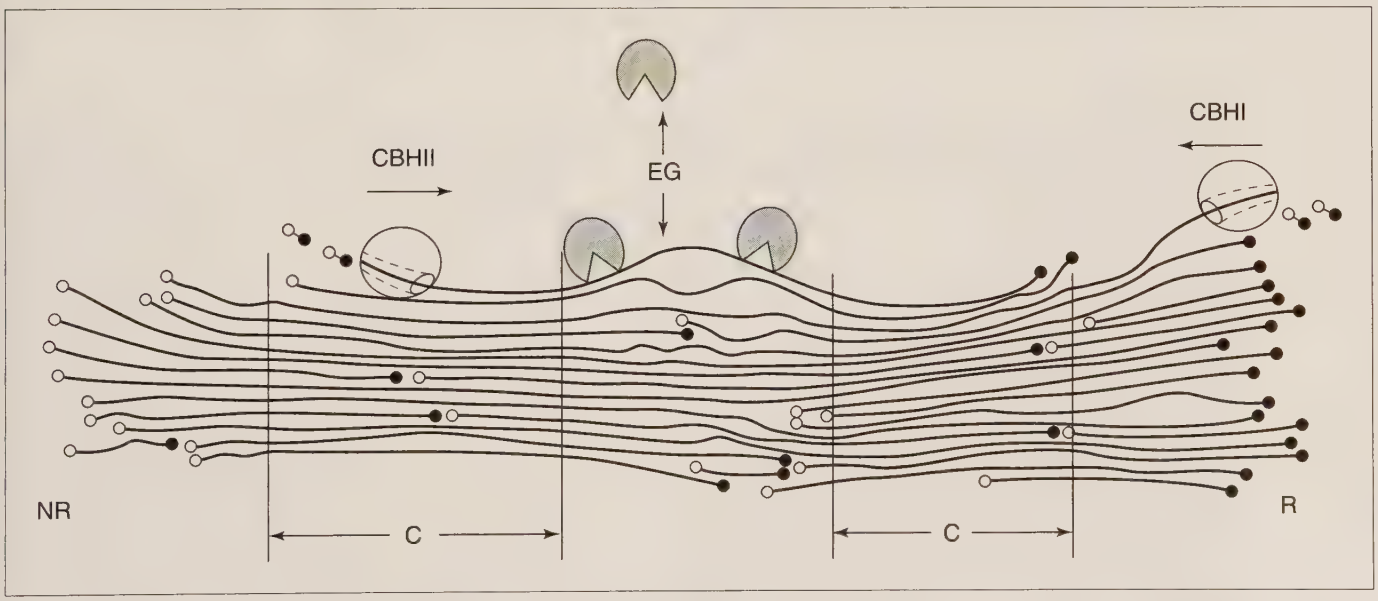

Figure 1

Schematic representation of the morphology and organization of crystalline cellulose as described in Ref. 3. Because cellulose is a natural polymer, its crystallinity is rarely perfect. Amorphous regions occur in particular near the crystal surfaces but may also transverse the entire width of the crystallite[7,9,10]. In addition, hidden chain ends not exposed on the crystal surfaces have been proposed to occur even in the highly crystalline regions of cellulose[3,7,8]. Different exoglucanases or cellobiohydrolases (CBH) are currently thought to attack the crystalline areas at the opposite chain ends and the endoglucanases (EG) in the middle of the more disordered regions of cellulose. The filled circles, denoted R, represent the reducing ends and the open circles, denoted NR, represent the nonreducing ends. C defines the highly ordered crystalline regions.

Box 1. Substrates and assays commonly used to detect cellulase 'specificities'

Soluble, substituted celluloses
Long cellulose polymers can be solubilized by hydroxyethyl (HEC) or carboxymethyl (CMC) substitution occurring at every two to five sugar units. Such a substrate is readily degraded by endoglucanases cleaving bonds between the substituent groups, resulting in a rapid decrease in the degree of polymerization (DP) and viscosity of the substrate. Strict exoglucanases seem to be limited to the hydrolysis of a few, unsubstituted sugar residues at the chain ends of CMC and HEC and do not decrease their DP or viscosity[15,16].

DP-dependent staining by Congo red
Congo red forms a red complex with oligosaccharides that contain at least five monosaccharides linked with β-1,3- or β-1,4-glycosidic bonds[17]. In plate or protein native gel assays, endoglucanase activity is visible as a clear zone around a colony or a protein band, where the substrate DP has been reduced and is therefore no longer stained by Congo red. An exoglucanase that does not decrease the substrate DP will not cause such a clearing.

Crystalline substrates
Soluble reducing ends, released to a larger extent by cellobiohydrolases than endoglucanases, have been measured after the hydrolysis of different polymeric substrates. More heterogeneous substrates such as filter paper, cotton and Avicel™ are suitable for the determination of total cellulolytic activities of cellulase mixtures or their synergistic interactions[15,18]. All these substrates differ somewhat in their DP, degree of crystallinity, amount of residual hemicellulose and other cell wall components, and morphology, and are therefore difficult to use for detailed studies of enzyme mechanisms. Acid-hydrolyzed, highly crystalline bacterial microcrystalline cellulose (BMCC) with a simple morphology has been used as a model substrate to study the mechanism of crystal erosion[19,20]. The DP of crystalline celluloses after enzymatic hydrolysis can be evaluated by gel permeation chromatography of the residual cellulose after solubilization[9,10]. Algal cell wall cellulose isolated from, for example, *Valonia macrophysa* has been used to visualize cellulase activity by electron microscopy[21,22].

Determination of soluble versus bound reducing chain ends
Filter paper or another insoluble cellulose can be used to determine the ratio of soluble versus substrate-associated reducing ends[23]. Endoglucanases make internal cuts on the surface chains of insoluble cellulose and produce both soluble and bound reducing ends. Exoglucanases release cellobiose from the ends of the cellulose chains and produce, primarily, soluble reducing ends.

Soluble oligosaccharides with a low DP
Cello-oligosaccharides with a DP of up to six glucose units are soluble in water. Oligosaccharides of different DP, sometimes coupled to chromophoric, fluorogenic or radioactive labels, have been used as model substrates to characterize the chemical reaction mechanisms of both endo- and exoglucanases[8,24–27]. However, data obtained on these substrates are of limited use in understanding the process of crystalline cellulose degradation, in which the efficiency of the enzyme action depends on its ability to access single glucan chains from a crystalline surface[28].

lead to synergy, i.e. enhancement of activity over the added activities of the individual enzymes. Synergy is rarely observed between two different endoglucanases, but is clearly apparent between certain exoglucanases[18,23,29,34–36]. The endo–exo synergy is easy to understand as the endoglucanases provide free chain ends on the cellulose surface for the exoglucanases to act upon[29]. Because the exo–exo synergy has been more difficult to explain in molecular terms, the possibility of low, inherent endoglucanase activity of the exoglucanases has been much discussed[11,17,37].

The presence of cellobiohydrolases is clearly the distinguishing feature of cellulase mixtures effective on native crystalline cellulose[23,38,39]. Recently, the combination of genetic engineering and structural biology, as well as a number of other biochemical and microscopic techniques, have provided novel insights into the modes of action of this important group of cellulases.

Two different classes of cellobiohydrolases

By sequence and structural comparison, cellulases and other glycoside hydrolases have been classified into families sharing similar structures and the same chemical reaction mechanism[11,14,40–43]. Potent cellulolytic fungi generally produce two different cellobiohydrolases (CBHs). In *Trichoderma reesei*, CBHI makes up 60% and CBHII 20% of the total cellulolytic protein, accounting for most of its cellulolytic activity. These two enzymes can achieve complete, although slow, solubilization of cellulose crystals even without the help of endoglucanases[21]. Corresponding cellulolytic enzyme systems from aerobic bacteria, such as *Cellulomonas fimi* and *Thermomonospora fusca*, produce exoglucanases similar to CBHII but seem to lack the homologue of the fungal CBHI (Table 1). Some anaerobic bacteria, such as several *Clostridia*, have evolved a different strategy for crystalline cellulose degradation. The enzymes produced by these bacteria form large protein complexes called cellulosomes (reviewed in Ref. 11), but even there the main component, Cel S, is an exoglucanase[48,49]. The second type of exoglucanase produced by the aerobic bacteria *C. fimi* and *Th. fusca* belongs to the same family as the Cel S of *Cl. thermocellum* (Table 1). In addition, the E4 of *Th. fusca* in family 9 also seems to behave like an

Table 1. Examples of cellobiohydrolases from different organisms

Organism	Enzyme	Family	Mechanism	End specificity	Refs
T. reesei	CBHII	6	Inverting	NR[a]	8,26,31,44
C. fimi	Cbh A	6	Inverting	NR	45,46
Th. fusca	E3	6	Inverting	NR	8,47
T. reesei	CBHI	7	Retaining	R[b]	8,25,32,44
Th. fusca	E4	9	Inverting	R	8,23
C. fimi	Cbh B	48	Inverting	R	17,46
Th. fusca	E6	48	Inverting	R	8
Cl. thermocellum	Cel S	48	nd[c]	nd	48,49

[a]Nonreducing end. [b]Reducing end. [c]nd, not determined.

exoglucanase, although with relatively high endoglucanase activity[8,23].

All cellobiohydrolases were long thought to liberate cellobiose from the nonreducing end of the cellulose glucan chains. However, an increasing body of evidence now indicates that different cellobiohydrolases have opposite chain-end specificities. Enzyme-kinetic data obtained using oligosaccharides labeled by ^{3}H at their reducing end or by ^{18}O at one glycosidic oxygen indicate that *T. reesei* CBHI, as well as *Th. fusca* exoglucanases E4 and E6, prefer the reducing end while CBHII and E3 act at the nonreducing end[8,25]. In addition, the two cellobiohydrolases of *C. fimi* have different end specificities[7,17]. For the *T. reesei* and *C. fimi* enzymes, additional data confirming their chain-end specificities have been obtained by NMR (Ref. 26) and by HPLC (Ref. 16) analyses of the products released from natural, unmodified cello-oligosaccharides. High-resolution structural data have revealed the orientations of many different oligosaccharide ligands in the active sites of both CBHI and CBHII, confirming their proposed end specificities (C. Divne and T. A. Jones, pers. commun; Refs 31,32). Furthermore, the combination of *T. reesei* CBHII and *Th. fusca* E3, both acting at the nonreducing end, leads to competition rather than synergy on crystalline cellulose[8,23].

Exoglucanases have tunnel-shaped active sites

Structure determination of the catalytic domains of *T. reesei* CBHII (Ref. 31) and CBHI (Ref. 32) has recently shed light on the molecular basis of their exoglucanase activities. Complex structures determined with different substrate analogues and inhibitors revealed active sites located in tunnels enclosed by two (CBHII) or four (CBHI) long loops (Fig. 2). The sequences corresponding to the active-site loops of exoglucanases are not present in homologous endoglucanases[31,32,50]. The structure of the *Th. fusca* endoglucanase E2, homologous to CBHII, confirms that one of the exoglucanase active-site loops is missing and the other adopts a conformation leading to an open active site in E2 (Ref. 50; Fig. 3). The same general features can be predicted from sequence comparisons of other endoglucanase–exoglucanase pairs, suggesting that their modes of action on polymeric substrates are dictated by the shape of their active sites. Endoglucanases with open active sites can bind to, and act in, the middle of the glucan chains while exoglucanases with tunnel-shaped active sites are confined to the chain ends. The finding that deletion of one of the active-site loops of the *Th. fusca* exoglucanase CbhA leads to an increased endoglucanase activity supports these ideas[51].

The active sites of *T. reesei* CBHI and CBHII are designed to bind a single glucose chain in a defined orientation, which is determined by protein–sugar interactions throughout the tunnels[31,32]. For both enzymes, cellobiose is the major reaction product released from the nonreducing end of the chain by CBHII and from the reducing end of the chain by CBHI (see above). Structure–function studies have identified six binding sites for glucose units in the active site of CBHII (Ref. 31; A. Koivula, PhD Thesis, University of Helsinki, 1996; Fig. 4) and suggest as many as ten subsites in the CBHI tunnel[32] (C. Divne and T. A. Jones, pers. commun). It is currently thought that a single glucan chain is fed into these tunnels from one end, followed by their threading through the entire length of the tunnel and subsequent bond cleavage and release of the cellobiose product from the far end of the tunnel[27,31–33]. After a single bond-cleavage event, the remaining chain end is still bound in the enzyme through interactions at a number of subsites (Fig. 4). This creates the conditions for a processive mode of action in which several consecutive hydrolytic events occur without dissociation of the enzyme and the substrate[14,31–33]. The fact that CBHII does not produce any cellotetraose during cellohexaose hydrolysis provides experimental support for such a processive mode of action[27].

Electron microscopic studies have shown that CBHII sharpens one end of *Valonia* algal cell wall cellulose crystals while CBHI yields narrower and subfibrillated crystalline elements[21,22]. It is easy to imagine that CBHII, with a shorter tunnel, occasionally falls off the substrate and reinitiates hydrolysis at an adjacent chain end, which leads to the apparent sharpening of the crystals[31]. CBHI, which has a much longer tunnel, would bind more tightly to the remaining glucan chain and be less likely to dissociate after each hydrolytic event[32]. Therefore, once bound to an available chain

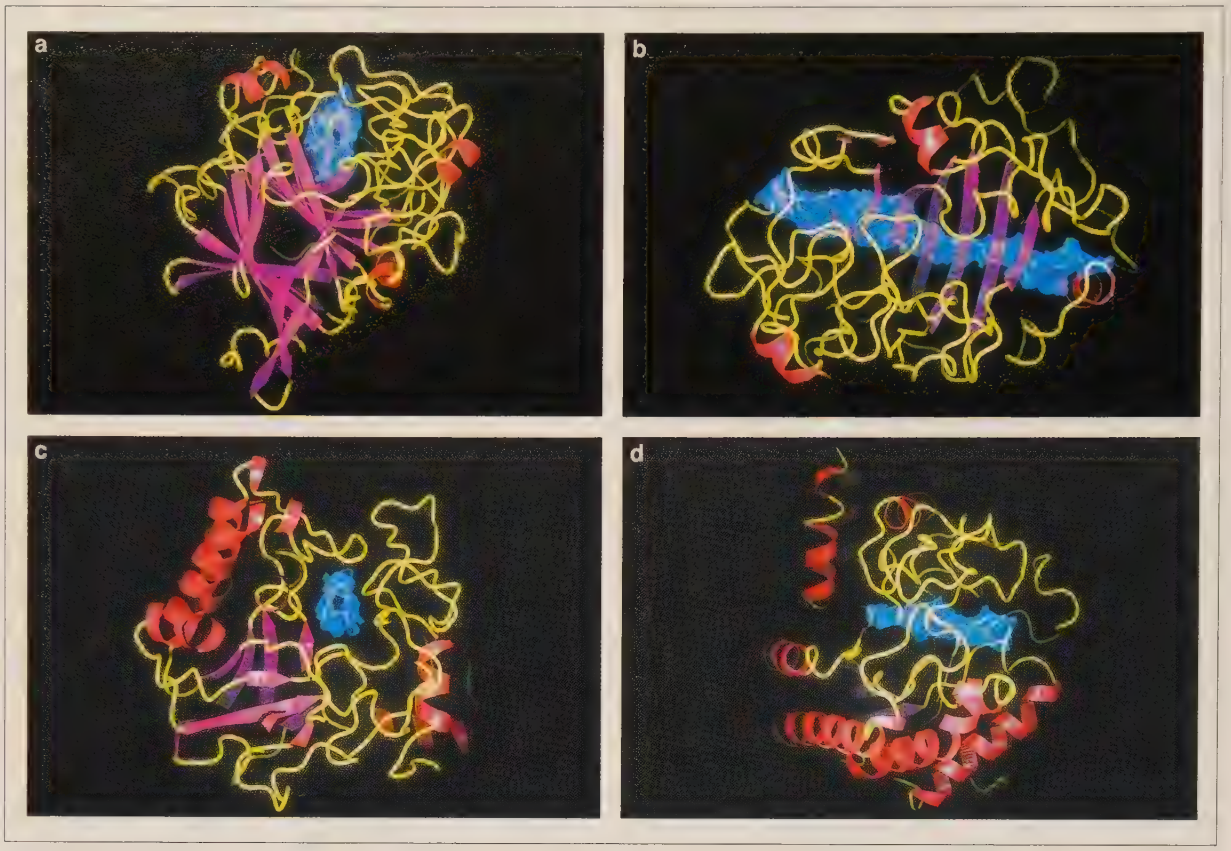

Figure 2

The α-carbon backbone traces of the structures of *Trichoderma reesei* CBHI (Ref. 32) (**a**) and (**b**), and CBHII (Ref. 31) (**c**) and (**d**). The secondary-structure elements in the proteins have been colored such that the helices are red, the β-strands purple and the loops yellow. The water-accessible surface of each active-site tunnel is indicated as a light-blue semitransparent lining. The view into the tunnel from one end shows how the tunnel is formed by surface loops in both CBHI (**a**) and CBHII (**c**). Viewing the tunnels from their sides shows that the tunnel in CBHI (**b**) is much longer than that of CBHII (**d**). (Courtesy of C. Divne, Uppsala University, Sweden.)

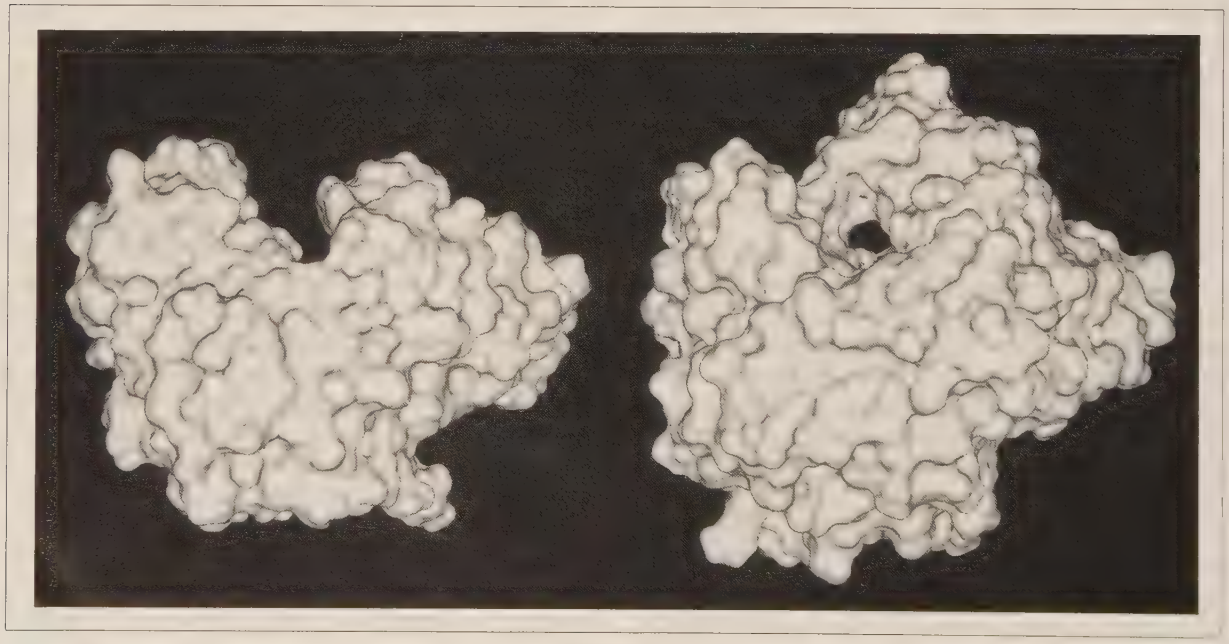

Figure 3

Computer graphic images of a homologous exoglucanase–endoglucanase pair to illustrate their different active-site topologies. Owing to the presence of two surface loops, *Trichoderma reesei* CBHII (Ref. 31; right) has a tunnel-shaped active site. The absence or different conformation of these loops in the *Thermomonospora fusca* endoglucanase E2 (Ref. 50; left) results in a more open active site, allowing easier access to internal glycosidic bonds. (Courtesy of B. Villoutreix, Technical Research Center of Finland.)

end, CBHI would continue hydrolyzing to the end. It is tempting to speculate that the thinning and lateral splitting of the crystals observed upon CBHI treatment results from the slow action of CBHI on a chain located within a cellulose crystal but with an exposed end for the initial binding of CBHI (see Fig. 1).

Concerning the exo–exo synergy, pretreatment of crystalline cellulose with CBHI has been shown to facilitate the function of CBHII and vice versa, even if the enzymes are not present simultaneously[36]. A cellobiohydrolase acting processively along the cellulose surface and peeling off single surface chains could induce disorder on the next layer of the chains on the cellulose crystal, thereby preparing an easier substrate for the following enzyme[32]. Owing to the discontinuities assumed to occur within the cellulose microfibrils (see Fig. 1)[3–8], a processive enzyme could also uncover new chain ends previously hidden under the surface, and together these phenomena could account for at least part of the synergy observed between CBHI and CBHII (Refs 7,8).

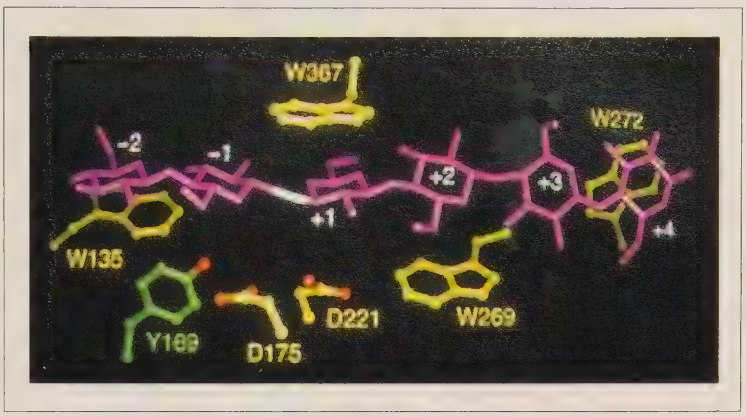

Figure 4

Schematic representation of a model of the binding of a single glucose chain (purple) into the active-site tunnel of *Trichoderma reesei* CBHII. Sugar residues from the site of bond cleavage towards the nonreducing end of the chain are numbered –1 and –2 and those towards the reducing end +1, +2, etc. The tryptophans W135, W367, W269 and W272 mediate sugar binding, and the residues D221, D175 and Y169 participate in the acid-catalyzed reactions of CBHII (Ref. 52). The chain is thought to enter the active site from one end of the tunnel, near tryptophan W272, and to thread through the tunnel until the nonreducing end is bound at the subsite –2. The β-1,4-glycosidic bond between subsites –1 and +1 (indicated in white) is then cleaved and the product, cellobiose, leaves from the far end of the tunnel[26,27,31,52]. After the release of cellobiose, the sugar remains bound to the CBHII active site by interactions at four subsites. In CBHI, there are even more subsites holding the sugar in the active-site tunnel after each bond cleavage[32,33]. (Courtesy of B. Villoutreix, Technical Research Center of Finland.)

Cellulases exhibit a continuum of overlapping specificities on crystalline cellulose

Owing to manifold experimental difficulties concerning both the substrates and the enzymes, undisputed classification of cellulases into endo- and exoglucanases has been difficult[11,17,35]. Although structural evidence for the entirely different active-site topologies of the endo- and exoglucanases has reconfirmed the validity of this classification, a question still awaiting experimental verification is whether the exoglucanase loops can occasionally open to allow initiation of the degradation at internal sites[28]. To date, of several different structures determined for CBHI and CBHII none have revealed significant conformational changes between the loops of native and ligand-bound enzymes (T. A. Jones, pers. commun; Refs 31–33). Furthermore, neither CBHI nor the *Th. fusca* E3 reduces the viscosity of CMC (Box 1), which is good evidence of both their purity and their exoglucanase nature[23]. This is confirmed by filter paper assays measuring the soluble reducing ends released by exoactivity in contrast to insoluble reducing ends produced by endoactivity (Box 1). Thus, both structural and biochemical evidence show that CBHI, E3 and E6 are exoacting enzymes producing, almost exclusively, soluble reducing ends. Considering the structural model of native cellulose, shown in Fig. 1, it can be argued that the insoluble reducing ends observed after the exoglucanase action[37] are those uncovered by the peeling action of the processive enzymes.

However, a loop that is totally disordered in the ligand-free structure adopts a well-defined structure enclosing the active site in a 'minitunnel' in the ligand-associated structure in the case of the endoglucanase EGV of *Humicola insolens*[53]. It therefore seems that, in response to the complexity and extreme heterogeneity of their natural substrate, cellulolytic enzyme systems have evolved to represent a continuum of activities, ranging from strict exoglucanases to very unspecific endoglucanases. In this continuum of activities, the strictest exoglucanases, such as CBHI, are confined to action at the chain ends by their long active-site tunnels, which are formed by many well-determined loops. Cellobiohydrolases similar to CBHII, which have shorter active-site tunnels, may exhibit increasing degrees of endoglucanase activity due to fewer loops with different lengths and/or mobilities[28]. Among endoglucanases, some might adopt a processive mode after an initial endo-attack, owing to loop-flipping motions enclosing the active site in a tunnel similar to those in the true processive exoglucanases. Finally, the 'classical' endoglucanases with fully-open active sites and non-processive activities at internal bonds along the cellulose chains are found in the far end of the enzyme system. As synergy has sometimes also been observed between certain endoglucanases, some differences in their modes of action must be anticipated. This continuum of activities seems to extend to the hydrolysis of other cell wall carbohydrates, and it is not unusual for cellulases to exhibit some activity towards xylan and other hemicelluloses[11,28].

Applied aspects

As a renewable and biodegradable polymer with exceptional mechanical strength, cellulose continues to be an interesting raw material for diverse industrial applications. Enzymatic processing of cellulose-based products is an increasingly attractive alternative to chemical processes[2], and crude mixtures of cellulolytic enzymes are already used, for example in grain and

fruit processing and in waste treatment applications. The textile industry uses somewhat-better-defined cellulase preparations for finishing textiles during manufacture and washing, and the paper industries experiment with cellulases to facilitate processing of pulps and recycled fibers[2,54]. In particular, cellobiohydrolases may find applications in specific fiber modifications and various surface treatments of paper and textiles since, unlike endoglucanases, they do not significantly affect fiber strength[2,30,54]. Enzymatic conversion of biomass to sugars that can be fermented to ethanol has been envisaged as a future source of fuels and chemicals, and cellobiohydrolases are clearly the key components of the redesigned, highly synergistic cellulase mixtures required for such processes[55].

The economic feasibility of industrial applications of enzymes is very much dependent on enzyme cost and performance[2,56]. In terms of production technology, low-cost enzymes can be produced by process optimization and genetic engineering of efficient fungal production strains[56,57]. Although the scale of many current and future applications of cellulases does not permit the use of purified enzymes, improved specificity can be achieved by using novel strains with enzyme production profiles tailored for specific applications[58,59]. However, to pick the right enzyme for each modification, the activities of the individual enzymes must be well characterized and, possibly, optimized by protein engineering. Recent leaps towards a better understanding of microbial cellobiohydrolases will help in both engineering their properties and improving their future biotechnical value. In this way, novel concepts can be developed for the enzymatic modification of cellulosic materials, perhaps facilitating a transition from bulk to speciality products based on cellulose.

Acknowledgements

T. A. Jones, M. Linder, M. Penner and D. B. Wilson are thanked for critical reading of the manuscript and R. Atalla, N. Gilkes, K. Kirk and J. Sugiyama for sharing their views and unpublished data on the structural details of crystalline cellulose. I am grateful to C. Divne and B. Villoutreix for preparing the computer graphic images of the enzymes and O. Lappalainen for drawing Fig. 1.

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HIV integrase: a target for AIDS therapeutics

Mark Thomas and Leo Brady

HIV integrase catalyses the incorporation of virally derived DNA into the human genome. This unique step in the virus life cycle provides a variety of points for intervention and hence is an attractive target for the development of new therapeutics for the treatment of AIDS. In this review we summarize current knowledge of the function of this enzyme and discuss some of the obstacles to the development of appropriate drugs.

Existing therapies for the treatment of AIDS are limited to inhibitors of the protease and reverse transcriptase (RT) enzymes of HIV. Resistance to these drugs develops rapidly through mutation of these enzymes, limiting their efficacy. Combination therapies, where two or more drugs targeted against one or both of these enzymes have been used simultaneously, appear to delay the onset of resistance. However, the long-term use of drug cocktails will require new drugs, and effectiveness seems to be maximized by inhibiting a variety of targets simultaneously.

A third viral enzyme, integrase (IN), catalyses the integration of the viral DNA into the host DNA, an essential step in the viral replicative cycle[1]. There is no known analogue for this enzyme in humans and so it is an ideal target for therapeutic intervention. Integrase-based therapies have, however, been slow to emerge, at least in part because of the experimental difficulties in obtaining useful structural details of this poorly soluble enzyme. Nonetheless, structural information is now starting to materialize and is providing a new basis from which to develop novel AIDS therapeutics. Much of our understanding of integrase structure, function and mechanism of action comes from studies of the homologous integrase enzyme from avian sarcoma virus (ASV) and comparisons of ASV and HIV integrases.

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The reactions catalysed

Integrase catalyses two temporally and spatially separated reactions known as *processing* and *strand transfer*. The first of these, processing (Fig. 1a), occurs in the cytoplasm where integrase cleaves a CA dinucleotide from the 3′-ends of the double-stranded viral DNA to leave a double-stranded DNA with a two-base overhang[2].

The protein–DNA complex is then transported into the nucleus where the strand transfer reaction takes place. This is a concerted cleavage–ligation reaction where the host DNA is cut leaving a 5′-overhang of five bases (Fig. 1b) and the 3′-ends of the viral DNA are covalently linked to the 5′-ends of the host DNA[3] (Fig. 1c). Repair of the 5-base gap between the 5′-ends of the viral DNA and the 3′-ends of the host DNA is achieved by host cell enzymes to yield the linear chromosomal DNA with the viral DNA insert (Fig. 1d).

In vitro, using synthetic substrates, a reversal of the joining reaction has also been observed[4]; this reaction, known as *disintegration*, has not been shown to occur *in vivo*. In all these reactions the active form of integrase is believed to be an oligomer, either a dimer or a tetramer.

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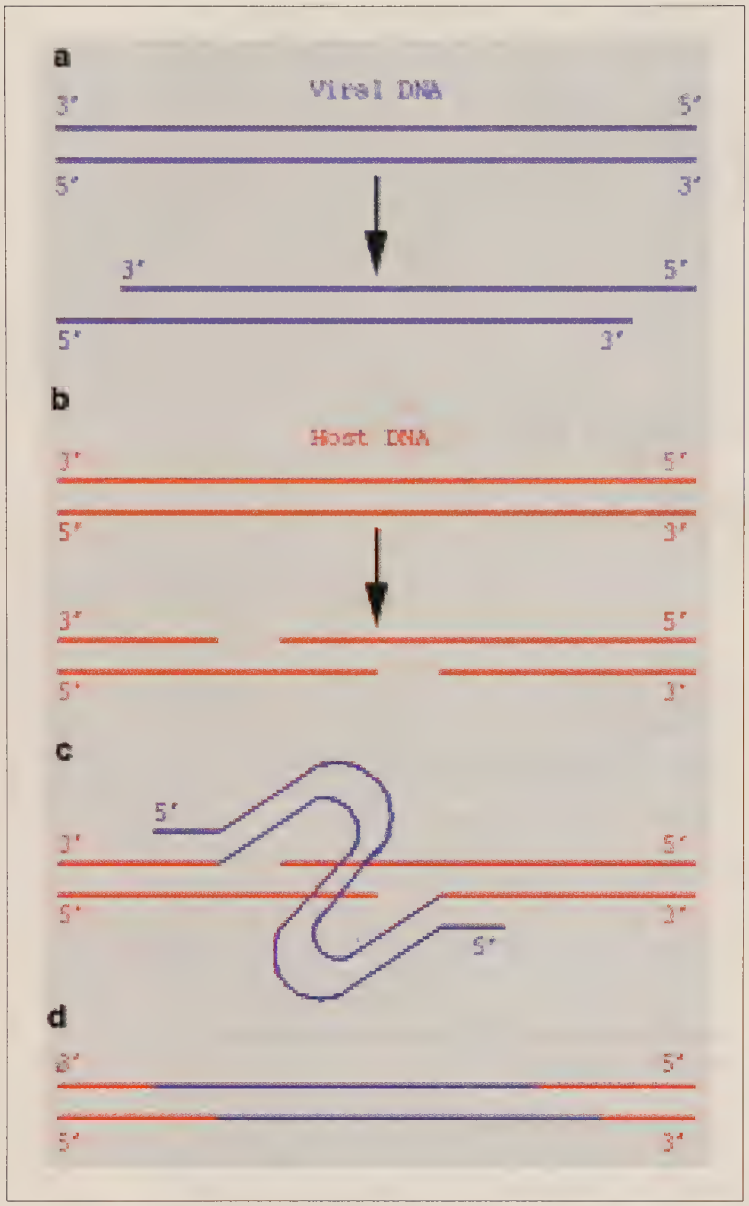

Figure 1

Reactions catalysed by HIV integrase. (**a**) Schematic showing the endonuclease cleavage reaction, or 3′-processing. The dinucleotide CA is cleaved from the 3′-ends of DNA encoding HIV and produced by reverse transcription of the viral RNA. In (**b**) and (**c**) the strand transfer, or integration, reaction is shown. Through a concerted reaction the target (host cell genomic) DNA is cleaved to provide an insertion site (**b**), and the 3′-processed ends of the viral DNA are ligated to the newly created 5′-ends of the host DNA (**c**). *In vitro* a further reaction, disintegration, can be observed, in which artificially created Y-shaped substrates corresponding to half of the structure in (**c**) are cleaved to produce two separate oligonucleotides. (**d**) The viral DNA integrated into the host DNA. Repair of the gaps left by the integration reaction is carried out by host cell enzymes.

Enzyme structure

The integrase from HIV-1 has 288 residues that fold into three domains, which have been identified by deletion studies[5]. Because of solubility difficulties when working with intact integrase, the structures of each of these domains have been pursued separately. There is very little known about the spatial arrangement of the three domains within a monomer[5], or assembly to higher-order species. Consequently, little detail is known about how the whole protein interacts with the DNA substrates.

The N-terminal domain

The structure of the N-terminal domain, comprising residues 1–49, is unknown, although an NMR analysis is currently underway. From the presence of an HHCC motif and an ability to bind Zn^{2+} (with the consequent assumption of an ordered structure) this domain is expected to adopt a zinc-finger conformation[6]. When expressed independently, the N-terminal domain of ASV does not bind DNA although a role in DNA binding in the full length protein is not ruled out[7]. It has been suggested that this domain plays a role in multimerization and aggregation of integrase monomers[8].

The C-terminal domain

The C-terminal domain comprises residues 213–288. It binds DNA non-specifically[7,9], is involved in multimerization[10] and contains a putative nuclear localization signal sequence[11]. The structure of residues 220–270, which form a dimer in solution, has been determined by NMR spectroscopy[12,13] (Fig. 2), but the significance of the SH3-like fold (similar to that found in signal transduction proteins) is not clear. The interface of the dimer is formed from β strands 2, 3 and 4, which are predominantly hydrophobic. β Strands 1 and 2 form a saddle-like groove that could accommodate a double-stranded DNA molecule, with the several basic side chains in the groove interacting with the DNA: mutation of one of these basic residues (Lys264) to Glu reduces the DNA-binding capability of this domain[14].

The core domain

The core domain (residues 50–212) includes those residues essential for catalysis. These residues, two aspartates and a glutamate, are arranged in a 'DD(35)E' motif where the latter two residues are separated by 35 amino acids. It has been proposed that these residues bind either one or two metal ions, Mg^{2+} or Mn^{2+}, that are essential for catalytic activity[7].

Crystal structures of the core domain of HIV-1 integrase[15] and ASV integrase[16] define integrase proteins as being members of a superfamily of polynucleotidyltransferases and nucleases[17] that includes bacteriophage Mu A transposase[18], *Escherichia coli* Ruv C resolvase[19] and RNases H from HIV-1[20] and *E. coli*[21]. The integrases have a mixed 5-stranded β sheet with three long antiparallel β strands (β1, β2 and β3) flanked by six (HIV) or five (ASV) α helices (Fig. 3). The catalytic residues are found in β1 [Asp64 (HIV integrase numbering)], a loop region between β2 and α2 (Asp116) and α4 (Glu152) such that the three side chains are in close proximity to each other. In the original published structure of HIV-1 integrase[15] the residues between β5 and α4, including Glu152, are disordered. However, it has subsequently been

reported that this region is partially ordered, albeit with high B-factors, in both HIV-1 integrase[22] and ASV integrase[16]. An overlay of the active sites of the two forms of integrase (Fig. 4) shows differences in the orientation of the catalytic residues, in particular Asp116, leading to speculation that the structure observed in the crystals of HIV integrase may not represent the active conformation[23]. This speculation is reinforced by the observation that Glu152 points away from the other two catalytic acids[22]. Further, metal binding in the active site has been demonstrated in crystals of ASV integrase[23], but not initially for HIV integrase. It seems likely that alterations in the conformation of catalytic residues may be necessary for the HIV crystal structure to achieve the active form, and this could make the formation of enzyme–inhibitor complexes difficult. Chemical modification of Cys65 (adjacent to the catalytic Asp64) by a component of the crystallization buffer (cacodylic acid)[15] in the HIV integrase crystals appears to lead to distortion of the Asp116 loop region, which then becomes stabilized by crystal contacts. To date, crystals in the absence of this modification have not been reported. Structures of complexes of integrase with DNA or inhibitors will be required before details of the active site configuration can be confirmed.

The core domains of both integrases crystallize as dimers with the primary contacts involving the α helices. The distance between the active sites is about 35 Å, which is too great to accommodate the five base pairs of B-DNA that separate the two HIV-1 IN cleavage sites in the target DNA. Possible solutions to this problem are: (1) two active sites could be positioned more closely in a higher-order multimer[15]; and (2) the reaction could proceed by partial unwinding of the DNA with co-ordinated but sequential cleavage at the target site. The second possibility is supported by independent evidence[24].

Therapeutic targets

Disruption of the activity of integrase as a means of therapy can, theoretically, be achieved in several ways. These include disrupting nuclear localization, dimer/multimer formation and the protein–DNA interaction, and inhibition of the processing and/or joining reactions.

Nuclear localization

To enable its transportation into the nucleus, integrase forms part of a pre-integration complex with the viral DNA and several other viral proteins[25], including the HIV matrix protein and RT, at least one of which also contains a nuclear localization signal sequence. To prevent nuclear localization requires that the whole pre-integration complex is confined to the cytoplasm or, to prevent integration, that the DNA is separated from integrase so that either the DNA or the integrase are prevented from entering the nucleus. Information regarding the pre-integration complex remains sparse.

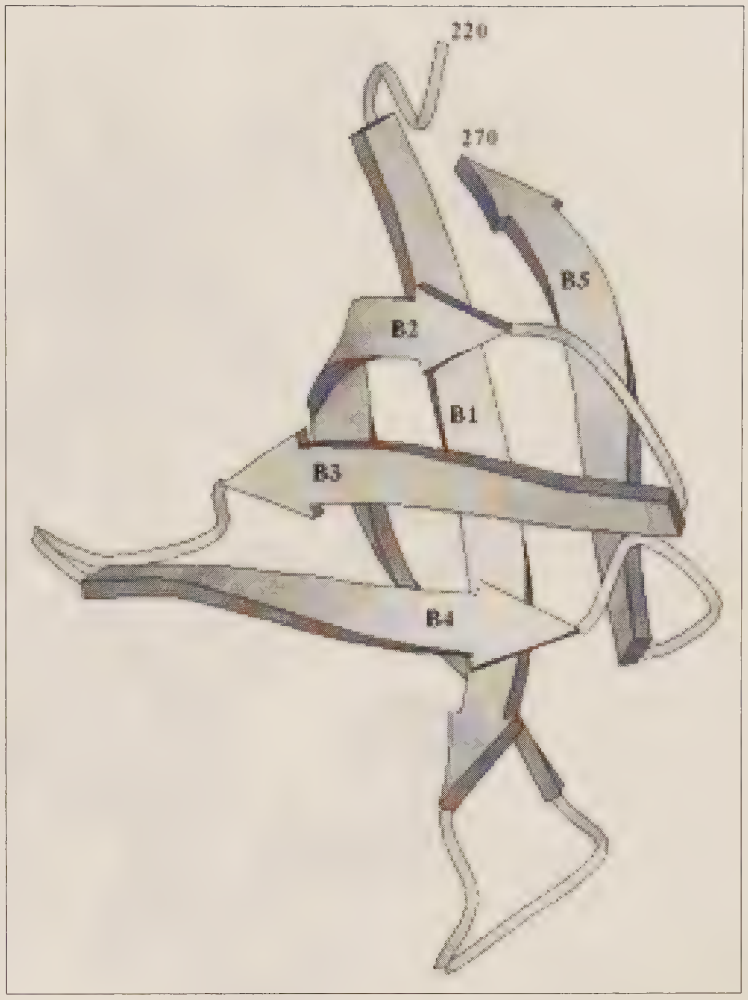

Figure 2
Schematic diagram (prepared using *Molscript*[34]) of the NMR-determined structure of the C-terminal domain of HIV integrase[12,13] (Brookhaven Protein Data Bank entry: 1IHW).

Protein–protein interactions

Integrase functions, minimally, as a dimer. Disruption of the interaction between integrase monomers would probably retard the integration reaction but targeting a specific disrupting agent to the cytoplasm or nucleus could be problematical. The dimerization surface observed in the structure of the core domain is largely flat and hydrophobic, presenting difficulties for targeting this site.

Protein–DNA interactions

Agents that disrupt protein–DNA interactions can do so by perturbing the DNA structure, changing the apparent surface of the DNA or by interacting with the DNA-binding sites of the protein. Perturbation of the DNA structure can be achieved with intercalating agents, and changing the surface of the DNA can be realized by chemical modification or by groove binders. Both approaches demand a high level of sequence specificity if the therapeutic agents are not to interfere with the normal function of host-cell DNA. Interaction of a therapeutic agent with the DNA-binding site(s) of integrase is probably the best

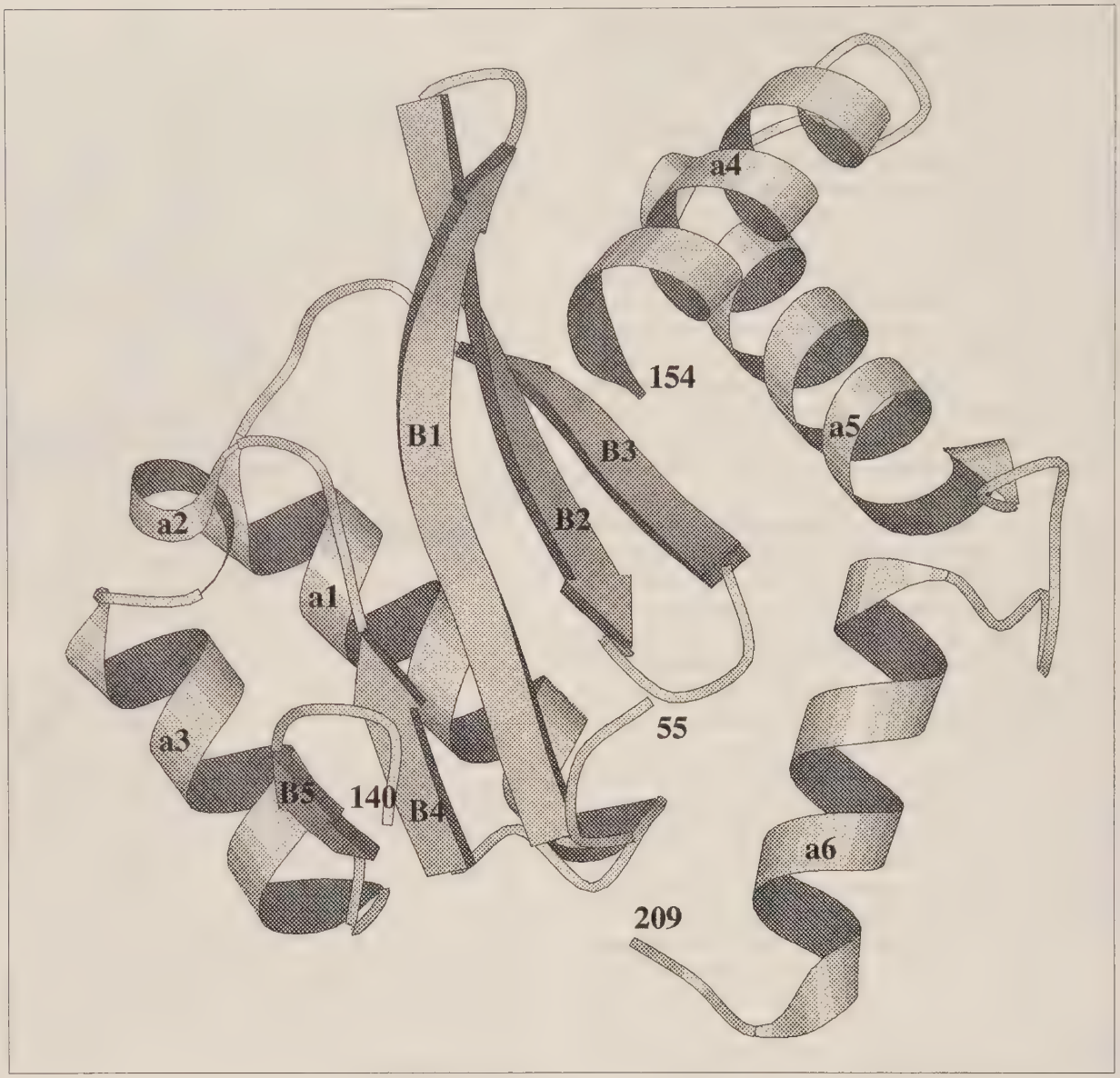

Figure 3

Schematic diagram (prepared using *Molscript*[34]) of the structure of the core domain of HIV integrase, as originally determined by Dyda *et al.*[15] (Brookhaven Protein Data Bank entry: 1ITG). The strands and helices are labelled. Residues 141 to 153 are missing as they are not ordered in the crystal structure.

approach to disrupting the protein–DNA interaction. For therapeutic purposes it may be possible to exploit the known sequence specificity of the enzyme. However, the structures of the enzyme bound to DNA fragments have not been determined.

3′-Azido-3′-deoxythymidine (AZT) is a licensed medicine for the treatment of AIDS. *In vivo* it is metabolized to produce the phosphorylated derivatives that inhibit RT. The monophosphate derivative inhibits the strand transfer reaction of integrase with an IC_{50} value of 140 μM (Ref. 26), which is significant because AZTMP accumulates in cells to concentrations of up to 1 mM (Ref. 27). The anti-HIV effect of AZT may, therefore, be due in part to the inhibition of integrase. It is possible that AZT-resistant strains of HIV may include mutations in the integrase protein to overcome the inhibitory effect of AZTMP.

The DNA at the ends of the viral DNA long terminal repeats is highly conserved in all HIV genomes, and so forms an attractive target for DNA-binding compounds. A heptapyrimidine (3′-CmeCmeCTTmeCmeC-5′) bearing an oxazolopyridocarbazole moiety has been shown to inhibit integration through the sequence-specific formation of a triple helix with the U3 long terminal repeat[28].

Processing and joining

Inhibition of either of these reactions would be sufficient to prevent integration. This requires a high degree of specificity to avoid inhibiting cellular DNA-binding proteins but advantage could be taken of the known specificity of integrase. A variety of compounds capable of inhibition have been identified.

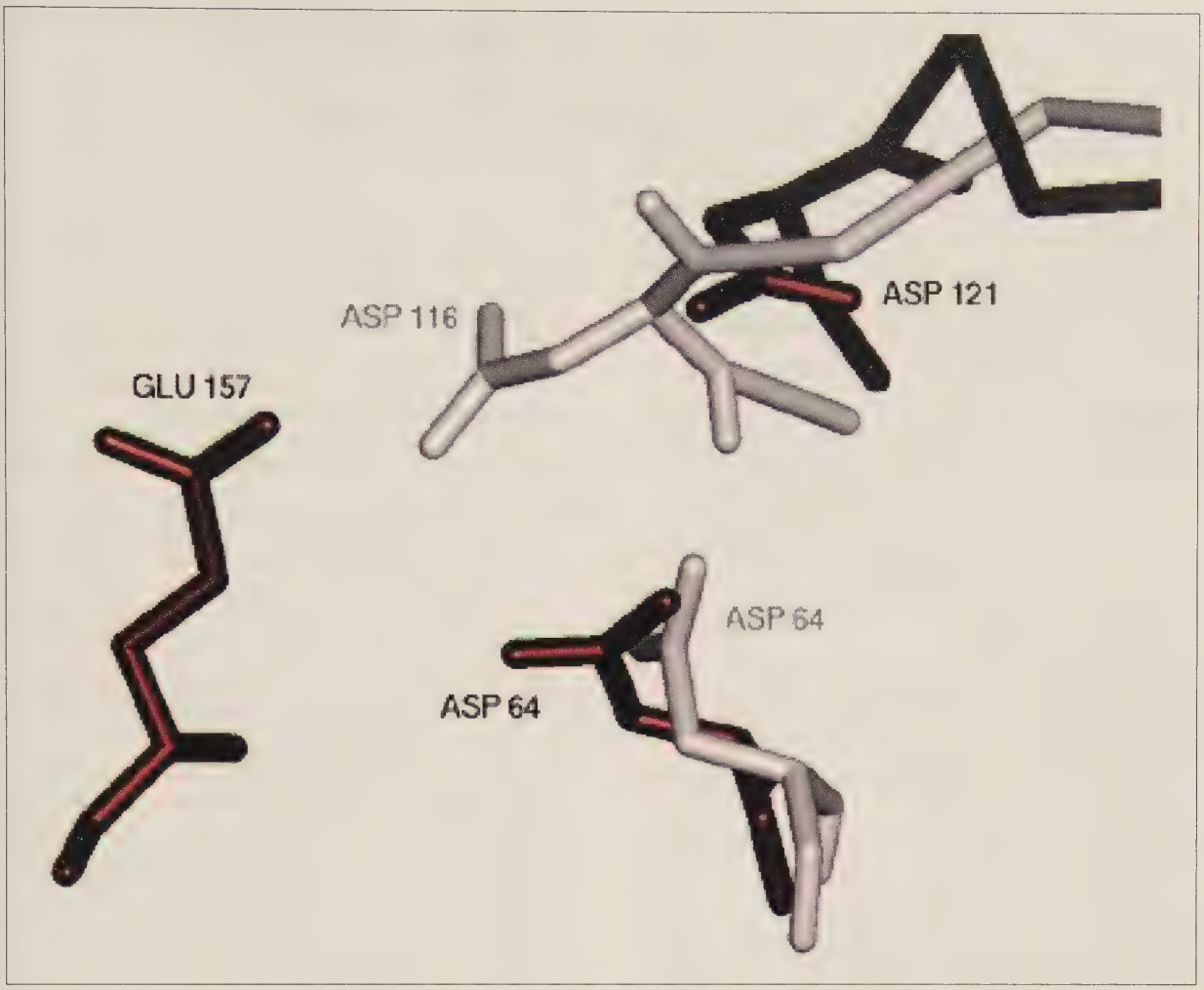

Figure 4

Overlay of the catalytic residues in HIV integrase (light) and corresponding residues from avian sarcoma virus (dark). Note that the conformation of the loop containing Asp116 in HIV integrase adopts a different conformation, which may be responsible for the altered side-chain arrangement of this residue. The glutamic acid of HIV (Glu152) is disordered and hence absent in the structure reported in Ref. 15 and this figure.

A series of studies by Pommier and co-workers have identified (2*R*,3*R*)-2,3-bis(3,4-dihydroxybenzyl)butyrolactone, a liganolide with two catechol substructures, as a potent inhibitor of both the processing and strand transfer reactions, with IC_{50} values of 21.4 ± 15.0 and 5.4 ± 4.0 μM, respectively[29]. As this compound can inhibit the disintegration carried out by the core domain only, it can be inferred that inhibition of processing and strand transfer proceeds by binding to the active site of the core domain.

Inhibition of the processing and strand transfer reactions, with an IC_{50} of 2 mM, by a hexapeptide (HCKFWW) has also been observed[30]. The hexapeptide also inhibits the core domain in the disintegration reaction, implying that it acts at or near the active site of integrase.

MAP30 and GAP31 are proteins from the plant species *Momordica charantia* and *Gelonium multiflorum*, respectively, that exhibit anti-HIV activity by apparently forming a complex with the enzyme[31]. Through stoichiometric inhibition of all integrase activities they are able to inhibit infection in T cells and monocytes as well as replication of the virus in already infected cells. Because of an inability to enter healthy cells these proteins are not toxic to normal uninfected cells. GAP31 has been crystallized[32] – elucidation of its structure may aid in the identification of those factors leading to the inhibition of integrase.

With the availability of the core domain crystal structure the way is now open for the rational design of inhibitors of the endonuclease and strand transfer reactions. A wide variety of compounds capable of inhibition have been reported, including curcumin, doxorubicin and quercetagetin, and are summarized in Ref. 33. Structures of the enzyme bound to any of these inhibitors, from which pharmacophores for structure-based drug design might be identified, have yet to emerge. It appears that the regions of disorder, the absence of adjacent domains, and, perhaps, the altered conformation of the active site in existing crystals have so far prevented discrete binding of a wide range of inhibitors. The discovery of new crystal forms, possibly from different integrase constructs, might enable these technical difficulties to be overcome.

Conclusions

Despite initial difficulties originating from solubility issues, there have been tremendous advances in our understanding of this unique enzyme over recent years. The complexity of integrase function provides many opportunities for intervention, ensuring the enzyme forms an attractive target for the development of novel AIDS therapies. Although significant technical difficulties continue to restrict structure-based drug design approaches, it seems likely that efficient use of the enzyme as a target for rational design will become possible in the near future.

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Progress and prospects for field use of *Bt* genes in crops

Marnix Peferoen

After ten years of development, genetically engineered crops expressing genes from *Bacillus thuringiensis* encoding insecticidal proteins are commercially available. *Bt* crops, for example *Bt* potatoes, cotton and corn, demonstrate high levels of protection from feeding damage by major insect pests, leading to a reduction in insecticide usage (e.g. *Bt* cotton) or a yield increase (e.g. *Bt* corn). Integrating *Bt* crops into traditional farming systems and preserving their performance by delaying resistance development in insect populations are the main challenges to be overcome.

Only ten years after the first demonstration that plants can be protected from insects by expressing a *Bacillus thuringiensis* (*Bt*) crystal protein gene[1,2], such crops have become available to growers. Engineering plants with a *B. thuringiensis* crystal protein gene was one of the first major projects of plant biotechnology. The value of such technology to the seed/biotechnology industry, the farmer, the environment and the consumer was evident. A quarter of all the pesticides used are insecticides and, by engineering an active insecticide into the crop, it was hoped that a substantial part of that insecticide market, estimated today at some US$7 billion, could be redirected from agrochemicals to the seed/biotechnology industry. *Bt* crops could reduce the cost, time and effort spent protecting crops from insects and could contribute to an environmentally friendly crop production system.

However, the journey from concept to product proved to be formidable. Transformation technology had to be developed for the successful transformation of commercial plant varieties, some of which proved amenable while others were recalcitrant. Hundreds of transgenic events were needed for the selection of the few lines with adequate expression of the transgenes while preserving the agronomic performance of the germplasm. Although the first crystal proteins had a valuable activity spectrum, additional proteins were required to target a wider range of insect pests and to develop strategies for insecticide resistance management. For several crops, expression levels of the crystal protein genes were too low and consequently the genes were modified in order to increase their expression. Major investments were made to build a forceful strategy to protect intellectual property rights, with patent applications on plant transformation, selectable markers, promoters, *Bt* crystal proteins, gene expression technology and resistance management. Patent offices were confronted with ethical issues concerning the patenting of 'life'. Crops expressing a bacterial insecticidal protein were totally new to regulatory agencies. Consequently, field trials were allowed to proceed only under very strict conditions and the registration process of such crops had to be defined.

In 1995, potato varieties expressing a *Bt* crystal protein toxic to the Colorado potato beetle were commercially available. In the following year, cotton varieties expressing a *Bt* crystal protein toxic to a number of lepidopteran pests, including the tobacco budworm and the cotton bollworm, were introduced. At the same time, *Bt* corn hybrids with improved resistance to the European corn borer (Fig. 1) became available to farmers. All these commercial introductions have taken place in the USA; *Bt* cotton varieties carrying the same *Bt* technology as used in the USA are being introduced in Australia. However, developments in Europe lag behind because of an unfavorable regulatory climate and moderate commercial interest in insect-resistant crops. The future of such crops will depend on their acceptance by growers, food processors and consumers. Industry (biotechnology, seed and agrochemical) will have to work with the producers to integrate this new technology into current farming practices and to promote its use.

Bacillus thuringiensis crystal proteins

Like many bacteria, under adverse conditions *B. thuringiensis* can suspend its vegetative growth and encapsulate in a spore. Typically, at the onset of sporulation, the *B. thuringiensis* bacterium starts the production of proteins that accumulate as crystals next to the spore. The spore can survive as such for several years, while the survival of the crystal is highly variable from a few days to several months, depending on environmental conditions. It is postulated that in nature, when

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PII: S0167-7799(97)01018-4

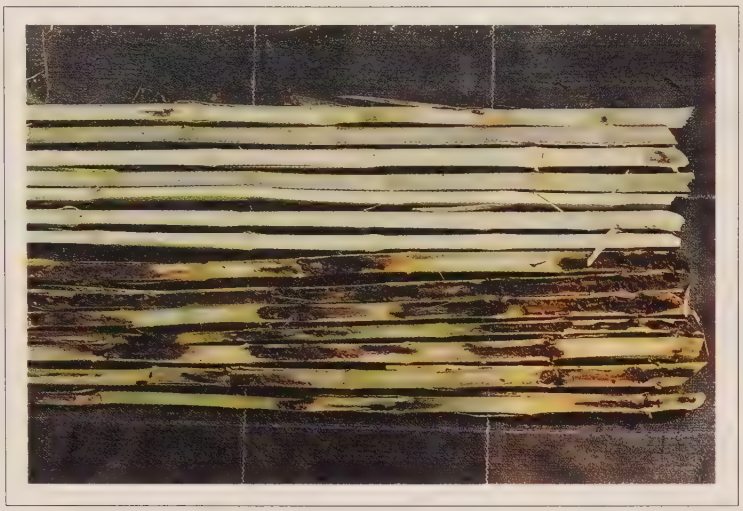

Figure 1
Corn plants were engineered to express the insecticidal part of the Cry9C crystal protein. Plants were infested in the field with European corn borer and at the end of the season, the level of protection from tunneling by the larvae was determined. Total tunnel length in control plants can be up to 40 cm per stalk. In the selected Cry9C corn plants, tunnel length is at most a few millimeters.

crystals and *Bt* spores are ingested by certain organisms, the crystal proteins destroy the gut wall and thereby create an ideal environment for germination and vegetative growth. Most *Bt* strains express different crystal proteins, each with its own spectrum of activity, thereby increasing the probability of being ingested by a host susceptible to at least one of the proteins[3].

In 1983 the first partial sequence of a crystal protein gene was published (see Ref. 4 for a review). Today more than 100 crystal protein gene sequences have been published, with some 60 proteins having less than 96% sequence identity, which can be further classified into 20 groups with less than 46% identity[5]. Each of the crystal proteins has a specific activity spectrum. For example, the Cry1Ab protein is highly active against the European corn borer and is used in current *Bt* corn hybrids[6,7]. The Cry1Ac protein is highly toxic to both tobacco budworm and cotton bollworm larvae and is expressed in the *Bt* cotton varieties commercialized in the USA and Australia[8], whilst the Cry3A protein is expressed in *Bt* potato varieties and provides protection from Colorado potato beetles[9]. In addition to the crystal proteins with a distinct activity spectrum, there are many crystal proteins that seem non-toxic when tested in bioassays with both insect and other invertebrate pests; indeed, only a few crystal proteins exhibit toxicity to important insect pests.

In addition to continuing interest in novel pesticidal proteins to control additional pests, the development of successful resistance management strategies requires a variety of pesticidal proteins, and *B. thuringiensis* remains one of the major sources of pesticidal proteins. Recently, it has been demonstrated that besides the crystal proteins, *Bt* also produces insecticidal proteins during vegetative growth[10]. Other bacteria such as *Clostridium bifermentans*[11] and *Bacillus popilliae*[12] have also been shown to produce insecticidal proteins and these could constitute a new source of pesticidal proteins.

Mechanism of action

The specificity of the crystal proteins for certain target organisms is to a large extent determined by the specific interaction of the protein with a receptor molecule on the cell membrane of the midgut epithelial cells[13,14]. Several studies indicate that aminopeptidase N, linked to the luminal side of the midgut epithelial cells, acts as a receptor for crystal proteins[15]. However, it is still unclear whether aminopeptidase N can act as a receptor on its own or whether other molecules are involved. Often insects susceptible to different crystal proteins have receptors specific for each crystal protein. Although binding is essential, toxicity appears to be determined by the formation of a pore in the cell membrane, which leads to cell lysis, disintegration of the midgut epithelium and finally death of the insect[16]. The mechanism of pore formation is poorly understood as it is unclear whether a single *Bt* protein molecule can puncture the cell membrane or whether an aggregation of several molecules is needed.

Since the elucidation of the general architecture of *Bt* crystal proteins, it has become possible to study the function of different structural domains of the protein[17] and to manipulate the activity and spectrum of crystal proteins. The crystal protein consists of three distinct domains (Fig. 2). Domain 1 appears as a bundle of α helices responsible for the pore formation activity of the protein[18], whilst loops at the apex of domain 2 seem to play a major role in receptor binding and the function of the third domain is presently unknown[19]. Using sequence alignments of crystal proteins of unknown structure with crystal proteins of known structure, together with molecular modeling programs, it has become possible to build structural models of crystal proteins. Such structural information has allowed directed mutagenesis studies. By exchanging corresponding domains between two crystal proteins, their insecticidal activity can be exchanged[20] or even be expanded[21]. By alanine scanning mutagenesis, it is possible to identify those amino acids in a certain domain that are key to its function. Random mutagenesis of such amino acids is expected to lead to the identification of mutants with improved activities. Consequently, together with traditional screening programs, mutagenesis studies to improve the binding affinity or pore-formation capacity are expected to result in crystal proteins with improved insecticidal activities.

From *Bt* plants to *Bt* crops

Crystal protein gene expression

In the early 1980s, when *Bt* crystal protein genes were being cloned and the stable transformation of plants using *Agrobacterium tumefaciens* was demonstrated, efforts were directed towards the engineering of insect protection into plants. However, the expression levels proved extremely low in plants transformed

with genes encoding the full length crystal protein, and such plants showed no significant insecticidal activity. Since it was known that the insecticidal activity of crystal proteins resided in the N-terminal half of the protein, transgenic plants were also generated with coding sequences for the insecticidal fragment of the crystal proteins. Transformations with such truncated crystal protein genes resulted in tobacco[1] and tomato[2] plants with significant levels of the insecticidal protein and that were protected from feeding damage by some pest insects such as tobacco hornworm, tobacco budworm and cotton bollworm larvae. Although such plants had a dramatic effect upon certain target insects, it became clear that for certain crops, the level of crystal protein had to be increased further in order to be commercially viable. While preserving the amino acid sequence, modifications were introduced in the crystal protein coding sequences, since it appeared that the coding sequence inhibited expression. The expression levels of crystal proteins have since been dramatically increased either by introducing limited modifications to specific regions of the coding sequence[22,23] or by resynthesizing the entire coding sequence[23].

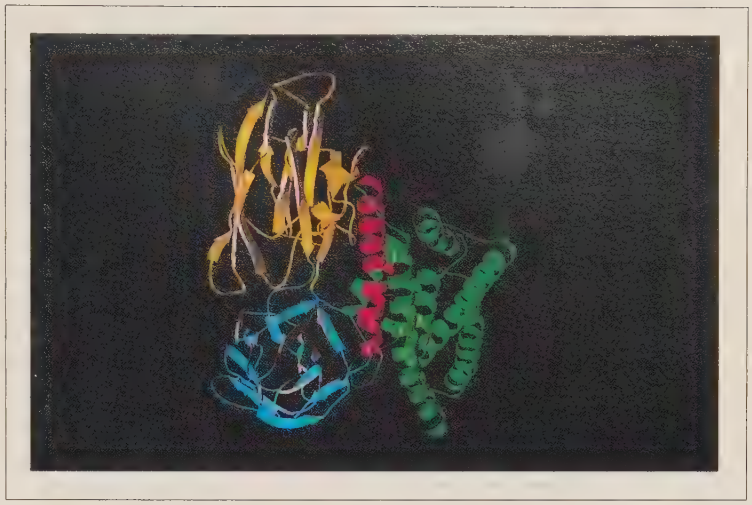

Figure 2

Crystal structure of the Cry1Aa insecticidal moiety (printed with kind permission of P. Grochulski[17]). The protein starts at its N-terminus with seven α helices, surrounding a central α helix; this is domain 1 and is responsible for the pore formation. The second domain consists of three β sheets with hairpin extensions and loops, postulated to be involved in receptor binding. A number of β sheets folded in a sandwich, with the C-terminus of the protein buried inside, form the third domain. These regions are conserved in different crystal proteins and constitute the core of the structure. This structure is believed to represent the general architecture of the toxic part of *Bt* crystal proteins.

Plant engineering

Over the last ten years, plant transformation technology has improved considerably. *A. tumefaciens*-based transformation have become a standard technique for plant molecular biology laboratories and is now routinely used to transform many dicotyledonous plants. In addition, the development of direct gene-transfer methods, such as electroporation and particle-gun bombardment, and the improvement of tissue culture methods has resulted in the stable transformation of corn, rice, wheat and various other monocotyledonous plants[24,25]. Often, direct gene-transfer methods result in multiple integrations of the transgenes and rearrangements of the heterologous DNA. Transgenes integrated in multiple copies appear to be more prone to silencing[26] and rearrangements complicate the molecular analysis of the transgenic lines. Recently, relatively high efficiency *Agrobacterium*-mediated transformations of rice[27] and corn[28] have been reported. Nevertheless, the key issue is transformation efficiency since there is a continuous flow of new transgenes and large numbers of transformation events are needed for the selection of the few lines with both the required transgene and agronomic performance. Transformation of elite germplasm is highly desirable since it obviates lengthy backcrossing procedures from a transgene in non-elite germplasm into commercial germplasm. The choice of the transformation protocol may also be influenced by intellectual property rights and regulatory issues.

Patents, regulations and business development

The development of plant engineering technologies requires substantial resources. Protection of intellectual property rights on these technologies is essential to secure the required investments. Some patents covering a broad field, for example a transgenic plant, a transformation method or a certain trait, have been granted and there are also patents on specific genes, gene expression strategies, selectable markers and promoters. Transgenic crops contain several technological inventions that are often covered by patents owned by different parties. Therefore, companies have to commit substantial efforts and resources to acquire rights to use certain technologies controlled by third parties. In the case of *Bt* crops, the multiple and complex intellectual property ownership issues have led to several litigation proceedings.

Regulatory agencies such as the Environmental Protection Agency consider *Bt* crops as pesticides. *Bt* as a sprayable product has been used on a significant scale since the early 1970s and its safety has been demonstrated[29]. It has been argued that expressing the same protein or part of that protein in another host may change its properties. Therefore, the registration process involves toxicological assays on a range of organisms such as mice, birds, earthworms and honey bees. The tests not only involve the protein as such, but also the protein as produced by the plant. Regulatory agencies are also concerned about the potential development of resistance to *Bt* crops by insect populations. Therefore, as part of the registration package, companies have to provide a resistance management strategy, which contains recommendations for the growers, monitoring protocols to analyze whether resistance is developing in the field and mitigation procedures. *Bt* corn expressing the Cry1Ab crystal protein in kernels has received approval with restrictions on acreage in regions where Cry1Ac cotton is grown. In these areas the transgenic corn could impose an additional selection pressure on corn earworms, also

known as the cotton bollworm, the main pest of cotton and targeted by the Cry1Ac cotton.

Since the *Bt* technology is 'carried' by the seed, commercialization strategies are based on the specifics of each crop. For hybrid crops such as corn, new seed is purchased each year, as saved seed that is replanted does not give the same yields as hybrid seeds. Therefore, the value of *Bt* incorporated in hybrid crops is protected and premiums for the technology can be added to the seed price. Conversely, for inbred crops such as cotton, farmers can save and replant the seed. However, seed is usually purchased from seed companies because it is of higher quality and its cost is only a fraction of the production costs. Nevertheless, a substantial increase in the price of the seed may prompt farmers to save and replant seed from inbred crops. In this case, the value of *Bt* is not recuperated through the seed but by considering it to be an insecticide. Farmers have the choice either to buy pesticides from agrochemical companies or to take a license from the biotechnology company to use *Bt* technology that allows them to buy and grow seeds containing a *Bt* protein.

Resistance management

The success of *Bt* crops will, to a large extent, depend on whether target pests develop resistance to them. Insects have developed resistance to almost all control measures that have so far been applied. Consequently, *Bt* crops that are being commercialized take into account the fact that the insect populations being targeted may have genes that can provide resistance to the *Bt* crops, and specific measures are being designed and implemented to minimize the build-up of resistance genes in insect populations.

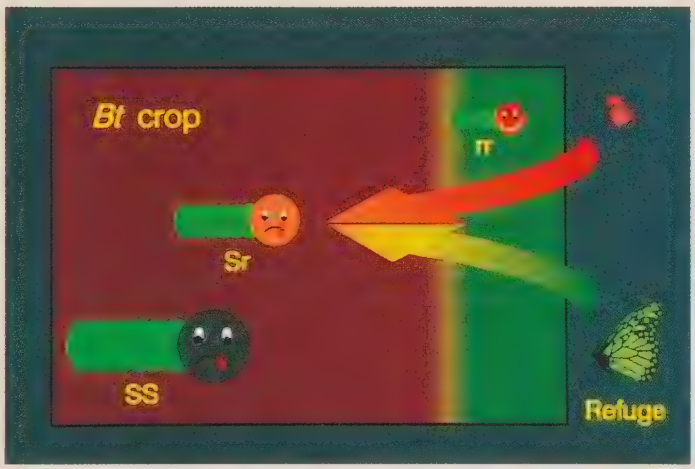

Figure 3

Schematic presentation of the high-dose/refuge resistance management strategy. In this example, the insect population carries a resistance allele that is nearly fully recessive, so that only homozygous resistant insects escape control by the *Bt* crop. Assuming that the resistance allele occurs at a frequency of 1 for every 1000 susceptible alleles, for every 1 million susceptible insects there would be some 2000 heterozygous resistant insects and 1 homozygous resistant insect. Homozygous resistant insects developing on the *Bt* crop could be forced to mate with susceptible insects maintained in a refuge near to the crop. Their offspring would be heterozygous and could be effectively controlled by the *Bt* crop. SS: homozygous susceptible, Sr: heterozygous resistant, rr: homozygous resistant.

Initially, *Bt* plants were perceived as more likely to enhance the development of resistance than *Bt* sprays, mainly because transgenic plants would provide a constant selection pressure on insect populations. Currently, the focus is on exploiting the unique features of *Bt* crops in designing strategies to retard resistance development[30]. There is very little empirical data and so computer models are being used to design strategies to delay or avoid the development of resistance in insect populations[31,32]. A consensus has developed on the 'high-dose/refuge' strategy, which aims to eliminate resistance genes and to dilute resistance developing in insect populations (Fig. 3). The most effective way to eliminate resistance genes is by targeting the heterozygous resistant insects, since in a population the large majority of resistance genes are in a heterozygous condition. Resistance to *Bt* proved to be recessive in all cases of resistance developed in the field and in most of the laboratory selected populations[33]. Therefore, heterozygous resistant insects are only slightly less susceptible than the fully susceptible insects and both can be effectively controlled by *Bt* plants. For the successful removal of the heterozygous resistant insects from a population, it is essential that the plants express a high dose of *Bt* protein during the entire season and for all the developmental stages that feed on the plant. If the levels of *Bt* protein decrease towards the end of the growing season, heterozygous resistant insects could survive and consequently increase the frequency of resistance genes in the following generation. If larvae can proceed through their first developmental stage before they move into the *Bt* crop, these older larvae, especially those with heterozygous resistance, could survive on the *Bt* plants, again increasing the resistance-gene frequency. However, by growing transgenic plants with high *Bt* expression levels, only the homozygous resistant insects are left in the field. Then by providing an area in the vicinity of the *Bt* crop where susceptible insects can survive, the mating between homozygous resistant individuals and susceptible insects can be promoted, leading to heterozygous offspring that are susceptible to the *Bt* crop. Consequently, effort is being focused on how to design a refuge that would force the mating of susceptible insects with homozygous resistant insects[34]. Again, there is no empirical data and theoretical models are being used to develop some guidelines on the size and positioning of such refuges. During the period when *Bt* crops are being introduced refuges will be plentiful since the total acreage sown is quite limited. However, it is expected that the acreage of *Bt* crops will increase and refuge areas will become more scarce and consequently will have to be deliberately established. It is clear that for each crop, pest and area there will be different requirements. The implementation of high-dose/refuge strategies will require a dedicated effort from the biotechnology/seed companies, extension workers and producers. Nonetheless, resistance management programs have been

implemented for *Bt* cotton and *Bt* corn that are being adopted by the producers.

Prospects

Currently, the focus of the development and commercial deployment of *Bt* is in arable crops such as cotton and corn. *Bt* cotton is being licensed directly to the growers in the USA and Australia and, with a potential application on over two million hectares in the USA and 400 000 hectares in Australia, the overall value in these markets is in the range of US$250 million. The first large-scale (some 800 000 hectares) production in the USA has tempered the euphoria around *Bt* cotton. It appears that an exceptionally high level of bollworms caused severe feeding damage and some insecticide spraying was required to supplement the control provided by the *Bt* gene; however, producers may still have benefitted financially. In the USA, *Bt* corn is expected to increase yields by approximately 5% in about one third of the 30 million hectares of hybrid corn, which represents a value of about US$350 million. New technologies are being developed to add to these first *Bt* products. Both cotton and corn engineered with combinations of different *Bt* crystal proteins for resistance management strategies are being developed and could be commercialized within the next five years. In addition, the insecticidal activity spectrum of these crops will be extended to include other, perhaps secondary, pests by introducing novel insecticidal proteins. However, the success of the technology will depend on how the *Bt* corn and *Bt* cotton integrate into the agricultural production systems. Typically, producers grow three to five different hybrid corn varieties. They will have to decide how many and which of these hybrids are to express the *Bt* gene.

Bt technology could be used in a wide variety of crops all over the world. However, only a few crops justify the very substantial investments required to develop a *Bt* crop. At a later stage, technology developed for crops such as cotton and corn could also be used in markets with lower or less secure returns. *Bt* could add significant value to vegetable crops, but this is a highly fragmented market with numerous cultivars, requiring extensive transformation or backcrossing efforts to introduce the transgenes into the relevant varieties.

Arguably, the greatest contribution of transgenic crops to a safer and more environmentally friendly crop protection system would be in the developing countries. Both cotton and rice are crops grown on a very large scale in developing countries and require substantial insecticidal treatments. However, in developing countries there are no systems in place that provide effective protection of the intellectual property rights on the technology and consequently industry is reluctant to enter these markets with engineered crops. Perhaps national or international biotechnology or agricultural institutes could implement the technology already available in the developed world, or could initiate their own programs. Besides having access to the technology, its cautious deployment is another main challenge. It has been accepted that until we know more about the development of resistance, the introduction of *Bt* crops should be accompanied by a resistance management strategy.

Bt crops are, and will contribute to, an effective, user- and consumer-friendly crop production system with an improved environmental compatibility. They are not a 'stand alone' product but will become integrated into current farming practices. However, to benefit from *Bt* crops, close collaboration between the seed/biotechnology industry, crop managers, extension workers and producers will be essential.

Acknowledgements

I thank the PGS Insect Control Team whose work has inspired this review.

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Transient suppression of macrophage functions by liposome-encapsulated drugs

Nico van Rooijen, Jeroen Bakker and Annemarie Sanders

Macrophages play an important role in host defense reactions, for example, by phagocytosis of particulate materials. This process also results in the rapid removal of targeting devices such as liposomes and adenovirus vectors and of non-autologous grafted cells and materials. Another aspect of macrophage function is their production and secretion of proinflammatory cytokines. Transient and organ-specific suppression of macrophage function by liposome-mediated manipulation has been shown to improve the efficacy of drug and gene targeting and to reduce the symptoms of inflammatory reactions.

From an evolutionary point of view, macrophages form an ancient cell population, representing the main host defense mechanism before the development of the immune system. During evolution, these cells have also acquired important functions in the regulation of humoral and cellular immune reactions (serving as accessory cells for the induction of acquired immunity against particulate antigens), and in the control of various nonphagocytic cells that participate in immune responses. Consequently, in mammals, macrophages are multifunctional cells[1], and can be found in nearly all organs and tissues of the body. Their direct precursors, the monocytes, are released in the circulation from the bone marrow. Via the circulation, these monocytes migrate into organs where they differentiate into resident macrophages.

Selective *in vivo* depletion of macrophages forms a generally accepted approach to study their various roles. For this purpose, we have developed a method based on the liposome-mediated intraphagocytic delivery of drugs such as clodronate and propamidine[2] that ultimately causes macrophages to die (reviewed in Ref. 3; see also Fig. 1): intracellular accumulation of the drugs disturbs the metabolism of the cells and activates their cell-death program, which results in apoptosis[4]. Using this approach, it has been confirmed that macrophages play a crucial role in host defense reactions in addition to their scavenger function.

The capability of macrophages to ingest and digest non-self (e.g. microorganisms) and altered-self (e.g. aged erythrocytes and apoptotic cells) particulate materials is an important mechanism of homeostasis. However, the same mechanism is responsible for removal of the bulk of administered targeting devices such as liposomes and adenovirus vectors, as well as non-autologous grafted cells such as human cells grafted into severe combined immunodeficient (*scid*) mice. Correspondingly, it has been shown that suppression of macrophage functions by liposome-encapsulated drugs improves the efficiency of such treatments.

Macrophages also play a key role in many inflammatory reactions. It is generally believed that their role is mediated by the production and secretion of proinflammatory cytokines, especially tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1), which, in turn, trigger the production and secretion of chemokines by nonphagocytic cells followed by the recruitment of inflammatory cells to the site of inflammation. It has been shown, in various models of inflammation, that suppression of macrophage function by liposome-encapsulated drugs attenuates the symptoms of inflammation.

This article discusses the transient suppression of macrophage functions by liposome-mediated manipulation and its possible beneficial and therapeutic effects.

Transient suppression of macrophage functions

Suppression of macrophage functions can be achieved by the physical depletion of macrophages from one or more tissues or organs, or by blocking or selective suppression of macrophage functions without the actual depletion of the cells. In both cases suppression of macrophage functions is transient. After physical depletion, bone-marrow-derived monocytes (macrophage precursors) migrate into the depleted

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PII: S0167-7799(97)01019-6

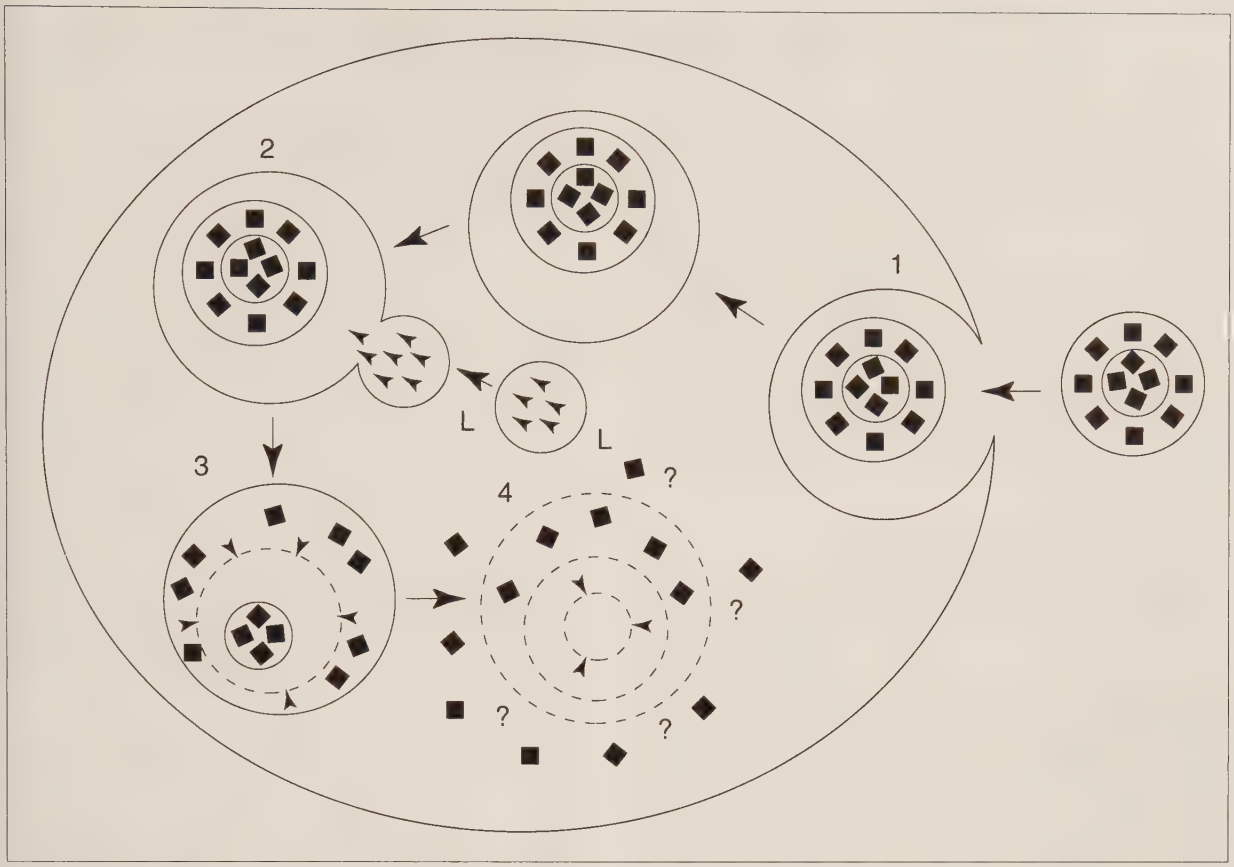

Figure 1

Liposomes, encapsulating drugs (■), are ingested by macrophages via endocytosis (**1**). After fusion of the endosomes with lysosomes (L) containing phospholipases arrowheads; **2**), the liposomal bilayers are disrupted (**3**) and drug molecules released within the lysosomal apparatus of the cell. Whether drug molecules are also released from the lysosomal compartments into the cytosol of the cell (**4**) may depend on the activity of encapsulated drugs and liposomal phospholipids.

tissues and differentiate into new macrophages. In most cases all depleted macrophages are replaced within two weeks, while the first cells reappear at the end of the first week. However, in the spleen[5,6] and lymph nodes[7] complete repopulation with all macrophage sub-populations may take more than a month.

Physical depletion of macrophages by liposome-encapsulated drugs ensures that all macrophage functions in the depleted organ are abrogated, and both the liposomes and the encapsulated drugs are inert materials that have a short half-life[3]. Furthermore, empty liposomes[8], free diamidines[9] and both free[10] and encapsulated[11,12] bisphosphonates inhibit rather than activate cytokine production by macrophages.

Like most other agents that are toxic for macrophages, silica blocks their phagocytic capability when administered in a high dose[13,14]; however, low doses can stimulate monocyte–macrophage lineage cells to produce IL-1 (Ref. 15), IL-6 (Ref. 16), TNF-α (Ref. 17) and nitric oxide (NO)[18]. Recent studies[19] have shown that blockade of Kupffer cells (macrophages in the liver) by gadolinium chloride ($GdCl_3$) produces a similar increase in serum activities of TNF-α and IL-6.

The main problem with concentration-dependent, opposite effects is that *in vivo* administration of particles will always result in a high concentration at one site and a low concentration at another site. For example, macrophages located between densely packed cells or collagen fibers (such as those in the white pulp of the spleen and in the paracortical fields of lymph nodes) and macrophages separated from the injected particles by vascular or other barriers, have no easy access to the injected particles.

Macrophages and drug targeting

During the past decade, several particulate drug-carrier devices have been proposed. Among these, liposomes may be considered the most versatile and promising drug-delivery system (for a recent review see Ref. 20). However, the high phagocytic capacity of tissue macrophages prevents the bulk of all kinds of particulate carriers, including liposomes, from reaching their targets. They are rapidly ingested and digested by macrophages and after intraphagocytic release of the encapsulated drugs, the latter may influence the macrophages more than the intended target cells (see, for example, Ref. 21). Several modifications of the original liposome formulations, such as the incorporation of amphipathic poly(ethylene glycol) conjugates in the liposomal bilayers, have been proposed, which are able to reduce the recognition of liposomes by

macrophages. Nevertheless, a large percentage of these so-called long-circulating liposomes will still be ingested by macrophages[22].

The depletion of macrophages by intravenous treatment with liposome-encapsulated clodronate significantly prolonged the circulation time of liposomes administered subsequently, even when the latter liposomes were long-circulating liposomes[23]. The (macrophage-dependent) clearance function of the liver with respect to liposomes was almost completely abrogated 24 h after injection of the clodronate liposomes, but this function gradually returned after two days[23]. These results indicate that the efficacy of drug targeting through the use of particulate carriers may be improved by transient suppression of macrophage functions before administration of the drug carrier.

Macrophages and gene transfer

Among the available vectors for gene transfer *in vivo*, replication-deficient recombinant adenovirus vectors are very efficient at transferring genes to target cells. They are easy to manipulate, infect many different types of cells, can be prepared at high titers and are capable of direct *in vivo* introduction of foreign genetic material in the body[24,25]. However, both the innate immune system and the acquired immune system may reduce the efficacy of this approach for gene transfer. Whereas the acquired immune response against the adenovirus vector, represented by T and B cell functions, requires some time for its induction, elements of the innate immune system immediately attack the adenovirus vectors upon their *in vivo* administration. Macrophages are important participants in both innate and acquired immunity against viruses. It has been confirmed that depletion of macrophages may have an exacerbating effect on the course of viral infection, evident as higher and extended viremia and accelerated development of disease and death[26].

Recent studies[27] have shown that depletion of Kupffer cells by liposome-encapsulated clodronate, before intravenous administration of an adenovirus vector, led to a higher input of recombinant adenoviral DNA to the liver, an absolute increase in transgene expression and a delayed clearance of both the vector DNA and transgene expression. One week after administration of the adenovirus vector, peak transgene expression was found to be enhanced about tenfold in the macrophage depleted animals[27]. One month after administration, expression in the animals treated with clodronate-liposomes was still 38% of this peak value, whereas control animals that had the adenovirus vector but no liposomes showed no detectable expression after two weeks[27]. The depletion of macrophages to improve the efficacy of adenovirus-vector-mediated gene transfer is now performed by several research groups, especially with respect to gene transfer to the lung, where alveolar macrophages can be depleted by intratracheal administration of clodronate liposomes (see below). We have no doubt that other particulate carriers used for gene transfer, such as cationic liposomes, will also see an improved efficacy when injected into animals pretreated with clodronate-liposomes.

Macrophages and cell or tissue grafts

Macrophages and the survival of human cells in immunodeficient mice

Immunodeficient mice are widely used to maintain xenogeneic grafts of human cells. In this way, the role of human cells in host defense mechanisms, pathological disorders and diseases, as well as in normal human hemopoietic and immunological processes, can be studied under *in vivo* conditions[28,29]. However, despite the absence of functional T and B cell mediated immunological activity against the injected or engrafted human cells in *scid* mice, such cells are subjected to some host resistance from elements of the innate immune system. Mice bearing the *scid* mutation retain a number of elements of the innate immune system. Phagocytosis by macrophages and cytotoxicity by natural killer (NK) cells are thought to form the core of the remaining resistance against the grafted human cells[30].

Several considerations suggested that the depletion of macrophages from the liver and spleen by intravenous administration of liposome-encapsulated clodronate, or from the liver, spleen and peritoneal cavity by intraperitoneal administration of liposome-encapsulated clodronate, would strongly enhance the survival of human cells after grafting into *scid* mice. First and foremost, macrophages form an important component of the innate immune system of the mouse. Furthermore, local macrophages play an essential role in the differentiation of highly activated organ-specific NK cells[31]. Finally, the liver, spleen and peritoneal cavity are the organs most frequently populated by the engrafted human cells[28]. For long-term experiments, repopulation of the macrophages in *scid* mice can be prevented by reinjections of liposome-encapsulated clodronate every five to seven days[30].

The effects of macrophage depletion in *scid* mice on the survival of injected human peripheral blood lymphocytes were investigated by Fraser *et al.*[30] Control *scid* mice had no detectable human cells within 72 h. However, animals treated with liposome-encapsulated clodronate maintained a large proportion of human cells in the peripheral blood and spleen. After simultaneous implantation of human fetal thymic and liver tissue in control *scid* mice (*scid*–hu Thy/Liv mice), production of phenotypically normal human T cells into the periphery is induced for prolonged periods, but the cells cleared rapidly. The *scid*–hu Thy/Liv mice injected with liposome-encapsulated clodronate showed a transient increase in human cell content in peripheral blood and a large accumulation of human cells in the white pulp compartments of the spleen. These results demonstrate that murine mononuclear phagocytic cells play a crucial role in human cell clearance. Another advantage of macrophage depletion in *scid* mice before grafting human cells has been

emphasized (W. Terpstra *et al.*, unpublished): the minimal graft size of normal and leukemic human hemopoietic cells in *scid* mice that results in an outgrowth of the human cells in the mouse bone marrow appeared to be about ten times smaller in macrophage-depleted *scid* mice. This considerable reduction of the minimal graft size greatly facilitates studies on subsets of human hemopoietic cells, which are not easy to obtain in large numbers.

Mice lacking the elements of acquired immunity (e.g. *scid* or NIHIII mice) could be made susceptible to the development of the human malaria parasite *Plasmodium falciparum* by the depletion of macrophages, followed by substitution of mice erythrocytes by human red blood cells infected with *P. falciparum*[32]. In view of the scarcity of animals able to harbor human parasites, this novel model offers new approaches for malaria research.

In conclusion, normal and leukemic human hemopoietic cells, as well as human red blood cells, show a greatly improved survival in macrophage-depleted *scid* mice. This clearly demonstrates the responsibility of macrophages for the remaining resistance against human cells of mice devoid of elements of the acquired immune system.

Macrophages and corneal allograft rejection

Corneal graft rejection is characterized by a massive infiltration of both T cells and macrophages. Macrophages are found in large numbers in rejected corneal grafts, suggesting a role for these cells in the rejection process. In rats treated postoperatively with subconjunctival injections of liposome-encapsulated clodronate at the time of transplantation and several times thereafter, grafts were not rejected during the maximum follow-up of 100 days[33]. Cellular infiltration in these grafts was clearly reduced and there was a strong reduction in neovascularization of the cornea. Corneal grafts in rats belonging to control groups that had received empty liposomes (containing phosphate-buffered saline only) and those that had been given no additional treatment were rejected within the usual period of 17 days. These results confirm that macrophages play a crucial role in corneal allograft rejection[33].

Macrophages and inflammatory reactions

Using the liposome-mediated macrophage 'suicide' approach in various studies on inflammatory reactions, it has been shown that macrophages play a key role in inflammation. For instance: (1) selective depletion of macrophages produced a significant inhibition of HSV-1-induced chorioretinitis[34]; (2) selective depletion of phagocytic synovial lining cells largely prevented inflammation (synovitis) during immune-complex-mediated[35] and collagen-type-II-induced arthritis[36] and significantly reduced the propagation and exacerbation of chronic synovitis in an established arthritic knee joint[36,37]; (3) depletion of macrophages suppressed the clinical signs of experimental allergic encephalomyelitis[38]; (4) depletion of macrophages attenuated symptoms and the mortality rate in a model of zymosan-induced systemic inflammation[39]; (5) depletion of macrophages considerably reduced endotoxin-induced mortality[40] and TNF-α production[41] in animals, and reduced endotoxin-mediated fever[42]; and (6) depletion of alveolar macrophages decreased neutrophil chemotaxis to *Pseudomonas* airspace infections[43].

It is generally believed that inflammatory agents stimulate macrophages to produce and secrete cytokines and/or chemokines. The latter, in turn, induce the recruitment of inflammatory cells, either directly or by stimulation of the chemokine production of other (nonphagocytic) cells in the area (Fig. 2).

Organ-specific macrophage suppression

Organ-specific depletion of macrophages can be achieved by varying the administration route of liposomes (Table 1).

Kupffer cells in the liver

Kupffer cells play a key role in the homeostatic function of the liver. They form the largest population of macrophages in the body, make up 30% of the hepatic nonparenchymal cell population (reviewed in Ref. 44) and have easy access to particulate materials in the circulation. Consequently, a large proportion of all intravenously administered particulate carriers used for drug targeting or gene transfer will be prematurely destroyed before they reach their targets. Therefore, transient blockade of phagocytosis by Kupffer cells might be an important factor to optimize in drug targeting, gene transfer and xenogeneic cell grafting.

Lipopolysaccharide (LPS) is an integral component of the outer membrane of Gram-negative bacteria and a major contributing factor in the initiation of generalized inflammatory processes (septic shock and multiple organ dysfunction syndrome; reviewed in Ref. 45), which are the main cause of death in intensive care departments. It is well-known that Kupffer cells are a major site for removal from the circulation of endotoxin, to which they respond by an excessive production of proinflammatory cytokines, which in turn mediate the lethal effects of LPS (reviewed in Ref. 46).

Using the liposome-mediated macrophage 'suicide' approach, it has been confirmed that Kupffer cells are responsible for more than 95% of the LPS-induced IL-1β, IL-6, IL-10 and IL-12 (p40) mRNA production and for 50–75% of the TNF-α mRNA production[47]. Consequently, transient suppression of Kupffer cell activity might have beneficial effects on endotoxin-mediated generalized disorders.

To influence Kupffer cells, liposomes should be injected intravenously (see Table 1). Although several subpopulations of splenic macrophages will also have access to these liposomes, their total numbers are considerably lower than those of Kupffer cells and, moreover, they require higher doses of liposome-encapsulated drugs to be affected[2].

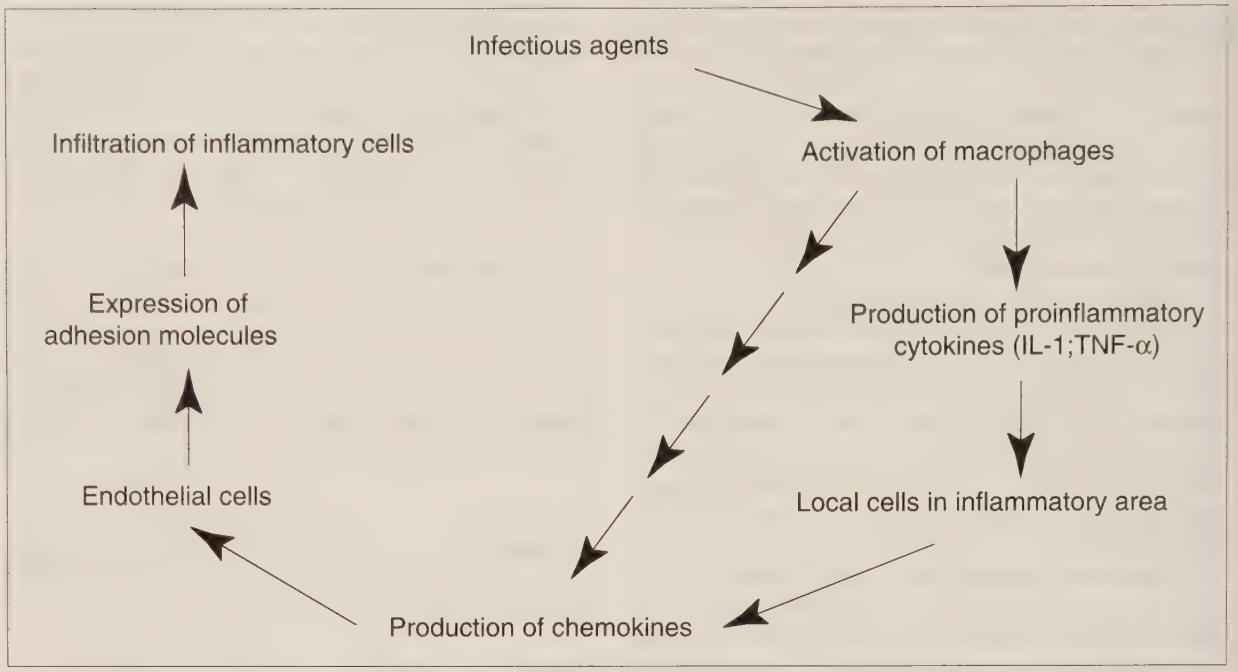

Figure 2

Cells and molecules involved in inflammation. As a result of activation by infectious agents, macrophages produce and secrete pro-inflammatory cytokines (TNF-α and IL-1). The latter then stimulate local cells in the inflammatory area to produce and secrete chemokines. Macrophages may also produce some chemokines. The chemokines stimulate the expression of adhesion molecules by endothelial cells in the inflammatory area, resulting in binding of inflammatory cells to the endothelial cells and their infiltration into the inflammatory area. Consequently, suppression of macrophage activity will reduce the inflammatory reaction. Abbreviations: IL-1, Interleukin 1; TNF-α, tumor necrosis factor α.

Table 1. Influence of administration routes on liposome-mediated depletion of macrophages

Organ/macrophages	iv	sc	it/in	loc	ia	ip	Model	Refs
Bone marrow macrophages	+	–	–	–	–	–	Rat	58
Knee joint/phagocytic synovial lining cells	–	–	–	–	+	–	Mouse	35–37
Liver/Kupffer cells	+	–	–	–	–	+	Rat Mouse	2 65
Lung/alveolar macrophages	–	–	+	–	–	–	Mouse Rat	48 43
Lymph nodes/subcapsular-sinus macrophages; medullary macrophages	–	+	–	–	–	–	Mouse	7
Peritoneal macrophages	–	–	–	–	–	+	Rat	53
Spleen/red pulp macrophages; marginal zone macrophages; marginal metallophilic macrophages	+	–	–	–	–	+	Mouse Rat	5 6
Testis macrophages	–	–	–	+	–	–	Rat	61
Thymus macrophages	–	–	–	+	–	–	Rat	57

Abbreviations: ia, intra-articular injection in synovial cavity; in, intranasal administration; ip, intraperitoneal injection; it, intratracheal instillation; iv, intravenous injection (in tail vein); loc, local administration in thymus or in testis; sc, subcutaneous injection (in draining area of lymph node); +, macrophage depletion induced by liposome-encapsulated clodronate.

Alveolar macrophages in the lung

Alveolar macrophages form a first line of defense against microorganisms entering the lung via the airways. In contrast to the interstitial macrophages in the lung, alveolar macrophages, which are located in the alveolar space, have direct access to liposomes administered via the airways, for instance by intratracheal instillation[48], intranasal administration[49] or by the application of aerosolized liposomes[43].

Adenovirus-vector-mediated gene transfer offers a direct method of correcting genetic pulmonary diseases and might also be used to correct temporary

abnormalities associated with acquired, nongenetic, disorders[50,51]. It is currently being investigated whether the transient suppression of alveolar macrophage function will improve the efficacy of the adenovirus-mediated gene transfer to lung tissues, similar to the improved gene transfer to liver tissue found after the blocking of phagocytosis by Kupffer cells[27].

Suppression of alveolar macrophage function has been shown to diminish neutrophil chemotaxis to air-space infections, an important aspect of inflammatory reactions[43]. As a consequence, transient suppression of alveolar macrophage function might relieve such inflammatory reactions in the lung. Antibody responses against antigens entering via the airways into macrophage-depleted lungs are enhanced when compared with those in normal lungs[48]. Therefore, the transient suppression or blockade of alveolar macrophage function might become part of a vaccination schedule in animals[3,52].

Peritoneal macrophages

For experiments in laboratory animals, liposomes are frequently administered along the intraperitoneal route. Such liposomes will primarily affect peritoneal macrophages. However, lymph drains from the peritoneal cavity towards the circulation and, as a consequence, liver and splenic macrophages will also be reached by intraperitoneally administered liposomes[53]. Thus, liposomes injected via the intraperitoneal route will affect higher numbers of macrophages than liposomes administered by other routes. This may explain why more animals are killed by intraperitoneal administration of clodronate-liposomes than by intravenous injections in cases where animals are contaminated with microorganisms, since macrophages play a key role in the clearance of all microorganisms.

Macrophages in lymphoid organs

One of the main functions of the spleen is its role in the induction of various types of immune reactions against blood-borne antigens. Consequently, splenic macrophages can be influenced by liposome-encapsulated drugs given along an intravenous route. Using the liposome-mediated macrophage 'suicide' approach, in combination with the finding that different subpopulations of splenic macrophages require different periods of time for their repopulation[5,6], functional differences between individual subpopulations can be investigated by the administration of antigens at various time intervals after liposome-encapsulated clodronate. This approach has produced evidence that: splenic marginal zone macrophages are involved in the induction of antibody responses against large particulate antigens such as foreign red blood cells[54]; splenic marginal metallophilic macrophages play a role in the response against thymus-dependent bacterial antigens[55]; and splenic red pulp macrophages are involved in antiviral cell-mediated immune responses[56].

Lymph nodes are involved in the induction of immune reactions against antigens in their draining areas. Macrophages lining the subcapsular sinus, as well as those located in the medulla of lymph nodes, can be influenced by subcutaneously administered liposomes[7]. Recently, it was shown that thymic macrophages can be affected by liposomes that were locally injected into the thymus[57], and bone marrow macrophages by intravenously injected liposomes[58].

Macrophages in arthritic knee joints

A single intra-articular injection with liposome-encapsulated clodronate in the mouse knee joint caused the selective depletion of phagocytic synovial lining cells (SLCs). Depletion was achieved within a few days, as studied by light microscopic, electron microscopic and immunohistochemical techniques, and a single injection of clodronate-liposomes eliminated phagocytic SLCs for at least four weeks[35,59]. Maximal depletion was observed on day 7 and gradual repopulation occurred in the following weeks. When an immune-complex-mediated experimental arthritis was elicited in SLC-depleted knee joints, inflammation was prevented. Joint swelling and the influx of inflammatory cells in SLC-depleted knee joints was highly reduced after elicitation with immune complexes, antigen or zymosan, as compared with observations in non-depleted joints. In addition, cartilage damage related to the influx of inflammatory cells was significantly reduced. The reduced influx of inflammatory cells was correlated to a decreased production of chemotactic factors. IL-1 levels were significantly reduced in SLC-depleted arthritic knee joints compared with the controls, and it has been postulated that IL-1-driven chemotactic factors may be involved in the normal influx of inflammatory cells. Further studies have shown that phagocytic synovial lining cells also play an important role in the propagation and exacerbation of smouldering synovitis[36,37]. The joint synovium surrounds a relatively small and separate area, requiring only a low dose of liposome-encapsulated drugs that will not easily reach other parts of the body. As a consequence, the application of liposomes in the therapy for certain forms of rheumatoid arthritis seems promising. The fact that free clodronate, released from liposomes or dead SLCs, might contribute to the suppression of arthritis[60] supports this opinion. The first small-scale human clinical trial is in preparation.

Macrophages in other tissues

The number of testicular macrophages was reduced by at least 90% by local injection of liposome-encapsulated clodronate into the testes of adult rats[61]. This approach has since been used in numerous studies on functional aspects of testicular macrophages[62,63]. However, in many organs or tissues, the local administration of liposomes will not influence local macrophages due to the fact that the distribution of liposomes from the injection site is hindered by a closely woven tissue structure (e.g. connective tissue) or by densely packed cells (e.g. lymphocytes in lymphoid organs).

Conclusions and perspectives

Since macrophages are present in nearly every organ of the body and play a role in many biomedical phenomena, research applications of the liposome-mediated macrophage 'suicide' approach are expected to increase.

Both clodronate[64] and defined liposome formulations[20] have been applied already in human studies and therapy, and therefore clinical applications of liposome-encapsulated clodronate, for instance in the therapy of certain forms of rheumatoid arthritis (see above), seem promising. When anti-inflammatory action is desired, the production of proinflammatory cytokines such as IL-1 and TNF-α should be markedly suppressed or completely abrogated to minimize the symptoms of inflammation.

Future applications might be in the prevention of phagocytosis of particulate drug or gene carriers, but a great deal of research remains to be done. Phagocytosis of the gene-carriers followed by intraphagocytic destruction of the genetic material can only be minimized by transient and local suppression of natural and acquired immunity against the carriers. The pretreatment of animals with liposome-encapsulated drugs to suppress the phagocytic component of natural immunity might well become a part of this strategy[27]. These studies have emphasized that merely a short-term blockade of phagocytosis is required at the time of administration of the gene-carrier. We expect that the blocking of phagocytosis for a period of time between some hours and one day will, in most cases, greatly enhance the efficacy of drug- or gene-delivery systems.

From a clinical point of view, the complete physical depletion of defined macrophage populations cannot be recommended because of the multifunctional character of macrophages and the release of cell remnants and molecules (from dead macrophages) in the circulation or lymph. One line of future research will focus on whether liposome-encapsulated drugs may be used for the selective and short-term blockade of phagocytosis or cytokine production without the physical depletion of macrophages.

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Bacterial surface display: trends and progress

Stefan Ståhl and Mathias Uhlén

Heterologous surface display on Gram-negative bacteria was first described a decade ago and is now an active research area. More recently, strategies for surface display on Gram-positive bacteria have also been devised and these carry some inherent advantages. Bacterial surface display has found a range of applications in the expression of various antigenic determinants, heterologous enzymes, single-chain antibodies, polyhistidyl tags and even entire peptide libraries. This article explains the basis of bacterial surface display and discusses current uses and possible future trends of this emerging technology.

The targeting and anchoring of heterologous proteins to the outer surface of yeast[1] and mammalian cells[2] has been utilized for various applications, and bacterial surface display is becoming an increasingly important research area[3–7]. Surface display of heterologous proteins in bacteria has been employed as a tool for fundamental and applied research in microbiology, molecular biology, vaccinology and biotechnology. The first examples of heterologous surface display were reported a decade ago[8–10], when short gene fragments were inserted into the genes for the *Escherichia coli* outer membrane proteins LamB, OmpA and PhoE, and the gene fusion products were found to be accessible on the outer surface of the recombinant bacteria. Since then, not only outer membrane proteins but also lipoproteins, fimbria proteins and flagellar proteins, as well as dedicated systems with coupled translocation and surface anchoring, have been employed to achieve heterologous surface display on Gram-negative bacteria[3–6], for example *E. coli* and *Salmonella* spp. More recently, systems have been described for heterologous surface display on Gram-positive bacteria such as staphylococci[11], streptococci[12,13] and mycobacteria[14].

The most common application of bacterial surface display has been in the development of live-bacterial-vaccine delivery systems because the cell-surface display of heterologous antigenic determinants has been considered advantageous for the induction of antigen-specific antibody responses when using live recombinant cells for immunization[5,14,15]. In this review, we also discuss the use of bacterial surface display in generating whole-cell bioadsorbents for environmental purposes[16], novel microbial biocatalysts (enzymes surface displayed with retained activity on, for example, *E. coli*[17,18] and staphylococci[19]), diagnostic tools (bacteria with surface-displayed antibody fragments[20–22])

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PII: S0167-7799(97)01034-2

and for the display of entire peptide libraries[23] (as an alternative to the rapidly developing phage-display technology). In addition, we describe novel techniques for the characterization and quantification of bacterial-surface-displayed proteins.

Surface display in Gram-negative bacteria

Display systems have been created for the expression of a number of heterologous proteins on the surface of Gram-negative bacteria (selected examples are presented in Table 1). Most often, foreign gene products have been fused to outer membrane proteins such as the maltoporin LamB, the phosphate-inducible porin PhoE and the outer membrane protein OmpA, and to lipoproteins such as the major lipoprotein Lpp, the TraT lipoprotein and the peptidoglycan-associated lipoprotein (PAL). Proteins from the filamentous structures present on Gram-negative bacteria have also been employed for surface-expression purposes, including fimbria proteins (such as the FimA protein[27] and the FimH adhesin[28] of type 1 fimbriae, and the F11 fimbrillin of P fimbriae[31]), the flagellar protein flagellin[26,35,36] and pili proteins (such as the PapA pilus subunit[34]).

Certain display systems are based on proteins that have specific mechanisms for translocation and surface anchoring. The lipoprotein pullulanase from *Klebsiella pneumoniae* has been employed for surface-display purposes in *E. coli*[32]. Using this system, the target protein becomes transiently anchored to the outer membrane by its N-terminal fatty acid moiety and is subsequently released into the culture medium. The β-domain of the *Neisseria gonorrhoeae* IgA protease precursor (Iga_β) and the *E. coli* AIDA-I have also been employed for surface exposure of various heterologous proteins[29,30]. When used as a C-terminal-fusion partner, the Iga_β and AIDA-I mediate attachment of hybrid proteins on the outer surface of *Salmonella*

Table 1. Examples of surface-display systems for Gram-negative bacteria with some areas of application

Display system (origin)	Host bacteria	Displayed protein	Comment	Refs
Surface display of antigenic determinants				
LamB (*E. coli*)	*E. coli*	C3 epitope of poliovirus	Positive immunolabelling on bacteria	8
PhoE (*E. coli*)	*E. coli*	VP1 of FMDV	Epitope recognized on bacteria by MAb	10
OmpA (*E. coli*/ *S. dysenteriae*)	*S. typhimurium*	Haemagglutinin of IAV	Partial protection in mice after oral immunization with live bacteria	24
TraT lipoprotein (*E. coli*)	*E. coli*	C3 epitope of poliovirus	Retained function of the TraT lipoprotein	25
Flagellin (*E. coli*)	*E. coli*	Hen-egg-lysozyme epitope	Functional flagella with accessible epitope	26
Fim A protein (*E. coli*)	*E. coli*	Epitopes from HBV, FMDV and poliovirus	Epitopes accessible at cell surface	27
Fim H adhesin (*E. coli*)	*E. coli*	PreS2 of HBV and CTB epitope	Epitopes accessible at cell surface	28
Iga_β (*N. gonorrhoeae*)	*E. coli*/ *S. typhimurium*	*Vibrio cholerae* CTB	CTB anchored at outer cell surface	29
AIDA-I (*E. coli*)	*E. coli*	*Vibrio cholerae* CTB	CTB anchored at outer cell surface	30
P fimbrillin (*E. coli*)	*E. coli*	Epitope from FMDV	Epitope recognized in fimbriae by MAb	31
Surface display of enzymes				
Pullulanase (*K. pneumoniae*)	*E. coli*	*E. coli* β-lactamase	Initial anchoring followed by slow release	32
Lpp–OmpA chimera (*E. coli*)	*E. coli*	*Cellulomonas fimi* cellulase	90% of hydrolase activity at bacterial surface	18
Surface display of antibody fragments				
PAL (*E. coli*)	*E. coli*	Chick-lysozyme-specific scFv	Surface accessibility demonstrated by IF	20
Lpp–OmpA chimera (*E. coli*)	*E. coli*	Digoxin-specific scFv	Enrichment by FACS of correct clones	21
Surface display of other proteins				
LamB/Pap pili (*E. coli*)	*E. coli*	SpA domains	IgG-binding activity at bacterial surface	33,34
Thioredoxin–flagellin (*E. coli*)	*E. coli*	Random 20-amino-acid library	MAb epitopes could be mapped by isolation of specific bacteria in a panning procedure	23
LamB (*E. coli*)	*E. coli*	Hexahistidyl peptides	Bacteria able to adsorb cadmium ions	16

Abbreviations: AIDA-I, adhesin involved in diffuse adherence; CTB, cholera toxin B subunit; FACS, fluorescence-activated cell sorting; FMDV, foot-and-mouth-disease virus; HBV, hepatitis B virus; IAV, influenza A virus; IF, immunofluorescence; Iga_β, immunoglobulin A protease precursor β; MAb, monoclonal antibody; OmpA, outer membrane protein A; PAL, peptidoglycan-associated lipoprotein; scFv, single-chain Fv antibody fragment; SpA, *S. aureus* protein A.

typhimurium and *E. coli* strains without the OmpT protease[29,30].

One particularly interesting system is based on the combined features of an Lpp–OmpA chimera[17]. The signal sequence and first nine amino acids of Lpp are fused to a region comprising three[37] or five[17,18] transmembrane regions of OmpA. This Lpp–OmpA display system has been shown to give efficient translocation and surface anchoring of the fused gene products, resulting in a high number of chimeric surface proteins present in an accessible form on *E. coli* cells[6,37].

Surface display in Gram-positive bacteria

Gram-positive bacteria have only recently been taken into consideration for bacterial surface display purposes[11,12]. A range of proteins, including antigenic determinants, heterologous enzymes and single-chain Fv (scFv) antibodies, have been targeted and anchored to the cell surface of Gram-positive bacteria (Table 2). Several Gram-positive bacterial hosts have been investigated in this context (Table 2), but until recently the research has focused on nonpathogenic staphylococci used in food-fermentation processes[47,48] (namely *Staphylococcus xylosus*[11] and *Staphylococcus carnosus*[40]), the mouth commensal *Streptococcus gordinii*[12] and attenuated mycobacteria[14]. Schneewind and co-workers finally elucidated the mechanisms of cell-surface targeting and subsequent anchoring of surface proteins on staphylococcal cells[49–52] several years after heterologous surface display had been achieved on staphylococci and streptococci[11,12]. They investigated how *Staphylococcus aureus* protein A (SpA) was sorted to the cell surface and suggested a highly plausible mechanism. The C-terminal surface-anchoring region of SpA (Fig. 1) consists of a charged repetitive region, postulated to interact with the peptidoglycan cell wall[53], followed by a region common to numerous cell-surface-bound receptors of Gram-positive bacteria containing an LPXTG motif, a hydrophobic region and a short charged tail[7,54]. It has been demonstrated that the latter tripartite region is required for cell-surface anchoring and that the cell-wall sorting is accompanied by proteolytic cleavage within the LPXTG motif, between the threonine and glycine residues, and subsequent covalent linking of the surface receptor to the cell wall[49–52]. The C-termini of numerous Gram-positive bacterial surface receptors are highly homologous[7,50,54], suggesting that this (or a similar) mechanism is utilized for their targeting to the cell surface.

The surface-display systems developed for *S. xylosus*[11] and *S. carnosus*[40] both take advantage of the cell-surface-anchoring regions of SpA, while the *S. gordinii* surface-display system[12] uses the similar C-terminal region of the M6 protein of *Streptococcus pyogenes* to achieve surface exposure of various chimeric surface proteins. While the staphylococcal systems utilize gene expression from plasmid vectors, the streptococcal system is based on incorporation of the target genes into the streptococcal chromosome by homologous recombination[11,55]. Recently, the *S. pyogenes* M6

Table 2. Gram-positive bacteria evaluated for surface-display applications

Bacteria	Display system	Displayed protein	Comments	Refs
Food-fermenting bacteria				
Staphylococcus xylosus	*S. aureus* protein A	Various antigens	Serum IgG after oral immunization of mice	11,15, 38,39
Staphylococcus carnosus	*S. aureus* protein A	Various antigens	Improved system compared with *S. xylosus*	39–41
S. xylosus/S. carnosus	*S. aureus* protein A	Anti-human-IgE scFv antibody	Whole cells able to bind antigen (human IgE)	22
Staphylococcus carnosus	*S. aureus* FnBPB	Enzymes: lipase/β-lactamase	Enzymes fully active at cell surface	19
Lactococcus lactis	*L. lactis* proteinase PrtP	Tetanus toxin fragment C	Antigen not accessible at outer cell surface	42
Commensal bacteria				
Streptococcus gordinii	*S. pyogenes* M6 protein	Various antigens	Serum IgG and mucosal IgA shown in mice	12,43–45
Sporulating bacteria				
Bacillus subtilis	*B. subtilis* CwbA	*Y. pseudotuberculosis* invasin	Immunization of mice with spores evokes antigen-specific antibodies	46
Attenuated pathogenic bacteria				
Mycobacterium bovis	*M. tuberculosis* membrane-associated lipoprotein	*Borrelia burgdorferi* OspA	Protective immunity evoked in mice by intraperitoneal immunization	14

Abbreviations: CwbA, cell-wall-bound autolysin modifier protein; FnBPB, fibronectin-binding protein B; OspA, outer surface protein A; scFv, single-chain Fv antibody fragment.

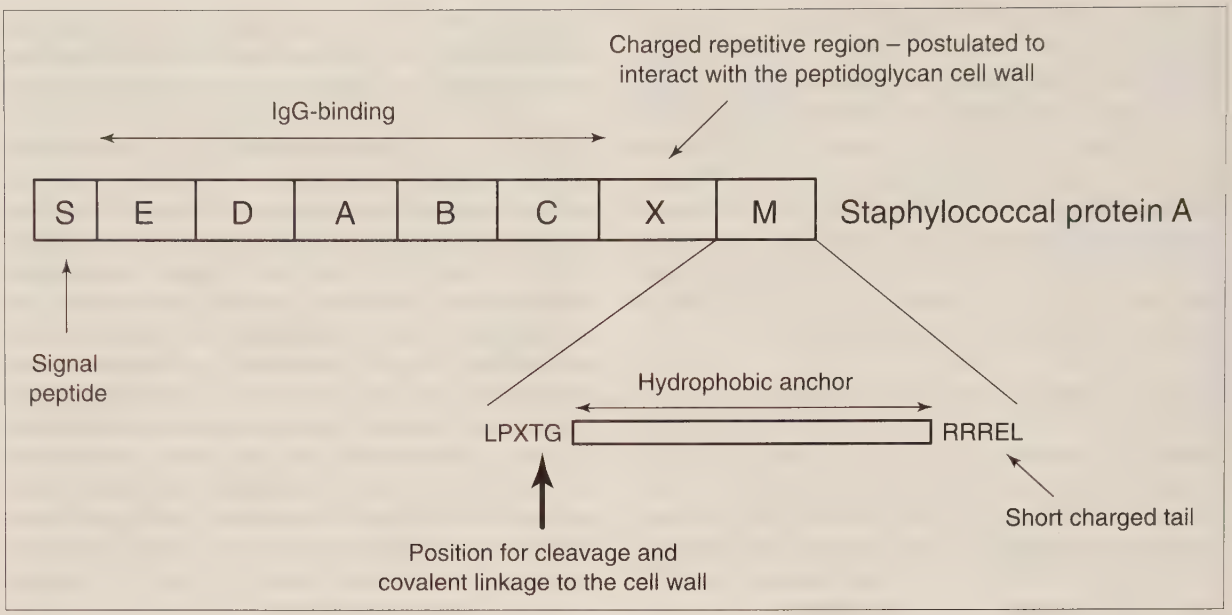

Figure 1

Schematic representation of the different regions of *S. aureus* protein A. The C-terminal regions X and M are used to achieve heterologous surface display. The M region contains the signals for covalent linkage of the chimeric surface protein to the outer bacterial surface[49–52].

protein was expressed on the surface of *Lactobacillus fermentum* and *Lactobacillus sake* (J-C. Piard, pers. commun), indicating the possibility of using lactic acid bacteria as host cells for surface display in the near future. In *Lactococcus lactis*, heterologous proteins have been targeted to the cell membrane by fusion to the C-terminal region of the cell-surface-associated proteinase PrtP (Ref. 42). However, chimeric proteins expressed by this system are not accessible on the outer cell surface[42]. To obtain surface association of heterologous proteins on the *Mycobacterium bovis* strain bacille Calmette-Guérin (BCG), relevant regions from a *Mycobacterium tuberculosis* membrane-associated lipoprotein were fused to the target protein[14]. Recently, heterologous surface expression was achieved in *Bacillus subtilis*[46] by fusion of the target protein to the cell-wall-bound autolysin modifier protein CwbA.

Powerful techniques to monitor and characterize the exposed target proteins are crucial. Traditional techniques for studying the recombinant surface proteins include immunofluorescence and immunogold methods[11] but, although these reveal whether a heterologous protein is produced in an accessible form or not, they fail to give reliable quantitative information about the number of exposed chimeric surface proteins per bacterial cell. Immunofluorescence staining can give misleading results by positive staining of partially lysed cells and should thus be accompanied by additional assays. Flow cytometry, in the form of fluorescence-activated cell sorting (FACS), has been used to characterize surface display on bacteria[14,15,40,41]. However, standard FACS technology does not give direct quantitative information concerning the number of exposed surface proteins. Recently, we (C. Andréoni *et al.*, unpublished) developed a novel technique for quantification of the number of chimeric surface proteins on bacteria. The method uses FACS technology and generates a calibration curve by using nonfluorescent plastic beads, similar in size to bacterial cells, coated with known amounts of monoclonal antibodies. Both the beads and the analysed staphylococcal cells were exposed to a fluorescein isothiocyanate (FITC)-labelled secondary antibody to allow FACS analysis. It was found that recombinant *S. carnosus* cells carried approximately 10^4 surface-displayed antigenic sites per cell (C. Andréoni *et al.*, unpublished).

Vaccine development

Many of the described Gram-negative systems have initially been evaluated in *E. coli* for surface display of various antigenic determinants, and subsequently applied to *Salmonella* spp. These species are of interest for the live, oral delivery of heterologous antigens for immunization[56]. Surface expression in *S. typhimurium* of malarial antigenic determinants using an OmpA system resulted in significant serum antibody responses upon oral immunization of mice[57]. The LamB system has been successfully used for surface display of heterologous epitopes in *E. coli*, producing epitope-specific antibody responses after intraperitoneal immunization of mice with live recombinant bacteria[58]. The system does not, however, seem to be as efficient for surface targeting in *Salmonella*[59,60]. It has been suggested that the *aroA* mutant strains of *Salmonella* frequently used for vaccine delivery are deficient in the translocation of LamB and its derivatives across the cytoplasmic membrane[59]. Immunizations of mice with live *Salmonella dublin* expressing a flagellum with an inserted cholera-toxin[36] or hepatitis-B-virus[35] epitope resulted in antigen-specific antibodies. Furthermore, it was found that in mice partial protection from influenza virus infection could be achieved through

immunization with live *S. dublin* carrying a haemagglutinin epitope from influenza virus in its flagellin[61].

The importance of having the antigenic determinants exposed on the surface of the bacteria when using *Salmonella* for live delivery of subunit vaccines is a subject of debate[62,63]. The initial antigen dose delivered by the recombinant bacteria has been shown to be important in priming the immune system[64], and a 10–100 times increase in the expression level of an antigen expressed in the periplasm is needed to achieve levels of antibodies similar to those induced by the same antigen expressed in a surface-exposed configuration[63]. It has been suggested that epitopes exposed at the bacterial cell surface induce antibody responses in a T-cell-independent manner[58] and that the lipopolysaccharides may serve as an adjuvant.

For the Gram-positive bacteria, research on the use of live bacteria to deliver surface-displayed antigenic determinants has focused on commensal or nonpathogenic bacteria[7,39], although investigations of attenuated pathogenic bacteria, such as mycobacteria[14], have been reported (Table 2). The staphylococcal host–vector systems based on *S. xylosus*[11] and *S. carnosus*[40] can efficiently target heterologous antigenic determinants to the surface of the cells. Both these bacteria are described as nonpathogenic[39,47] and are being used in starter cultures for meat-fermentation applications[47,48]. Both systems have been evaluated for the live bacterial delivery of many immunogens of diverse origin[11,15,38–41]. Oral immunization of mice with live *S. xylosus* carrying a heterologous surface protein comprising a region from streptococcal protein G and a trimerized epitope from respiratory syncytial virus (RSV) elicited serum antibodies to the hybrid surface protein, suggesting display of heterologous epitopes on *S. xylosus* as a potential delivery system for oral vaccination[38]. It has been demonstrated that surface exposure of the antigenic determinants is required with these systems in order to elicit antibody responses[15]. Both systems have proved to be nonpathogenic in mice upon oral or subcutaneous administration[39]. The *S. carnosus* system induced slightly higher serum antibody titres to a surface-displayed model antigen after subcutaneous administration than did the *S. xylosus* system[39]. This could potentially be explained by the higher number of recombinant surface proteins present on the *S. carnosus* cells[41]. *S. carnosus* has the additional advantage of longer persistence (more than 70 h) in the mouse gut[39]. Significant systemic antibody responses were recently reported to surface-exposed model antigens after oral delivery with the *S. carnosus* system[39].

The *S. gordinii* system has also been widely investigated for surface display of heterologous antigens, such as epitopes from papilloma virus and human immunodeficiency virus 1, and a protein allergen from the white-faced hornet[12,43–45]. Oral immunization of mice with the recombinant streptococci has been reported to result in serum IgG responses and significant increases in antigen-specific lung and salivary IgA[12,43–45].

Recombinant BCG carrying a surface-exposed antigen from *Borrelia burgdorferi* was able to evoke protective immunity to *Borrelia* infection in mice[14]. When the same antigen was expressed in either secreted or intracellular form, the corresponding recombinant BCG failed to evoke complete protective immunity[14]. *L. lactis* bacteria expressing the tetanus-toxin fragment C in a membrane-associated (but not surface exposed) configuration were significantly more immunogenic than those encoding secreted or intracellular versions of the same antigen[43]. It can thus be concluded that several of the Gram-positive bacteria are interesting candidates for the delivery of surface displayed heterologous antigens and that the surface association seems to be beneficial.

Other application areas

Surface display of enzymes

Certain enzymes have been expressed with retained activity on the surface of Gram-negative and Gram-positive bacteria (Tables 1 and 2) and the potential use of such recombinant bacteria as novel microbial biocatalysts has been discussed. *E. coli* β-lactamase, which is normally a periplasmic enzyme, has been successfully exposed on the outer surface of *E. coli* using the pullulanase system[32] or the Lpp–OmpA system[17], and the Cex exoglucanase of *Cellulomonas fimi* has been displayed on the surface of *E. coli* using the Lpp–OmpA system[18]. However, not all enzymes can be efficiently exposed on the bacterial surface. Alkaline phosphatase, which is normally periplasmic, was found to be retained in the periplasm of *E. coli* when expressed via the Lpp–OmpA system[65]. The efficacy of using *E. coli* with surface-displayed heterologous enzymes as novel microbial biocatalysts remains to be seen. Surface expression is undoubtedly an inexpensive way to produce immobilized enzymes, but Gram-negative bacteria might suffer from practical drawbacks such as cell lysis. Recently, a lipase from *Staphylococcus hyicus* and *E. coli* β-lactamase were expressed on the outer cell surface of *S. carnosus* with retained activity[19] (Table 2). Approximately 10 000 enzyme molecules were found to be present on each cell[19], and it was suggested that the rigid structure of Gram-positive bacteria would make them particularly appropriate as microbial catalysts[19].

Surface display of antibody fragments and peptide libraries

The expression of functional single-chain antibodies on the surface of *E. coli*[20,21] and staphylococci[22] has opened discussion on whether this strategy would be a way to create inexpensive diagnostic tools or alternatives to the rapidly developing phage technology for the selection of peptides or recombinant antibody fragments from large libraries[4–6]. A random peptide library was recently expressed in a conformationally constrained thioredoxin region, introduced into the flagellin gene of *E. coli* and thus surface-exposed into the *E. coli* flagellum[23]. The epitopes for three different immobilized monoclonal antibodies were mapped by

the selection of bacterial clones in a panning procedure, and the identified consensus epitopes had several amino acids in common with motifs found in the original antigens used to generate the monoclonal antibodies[23]. In addition, active single-chain antibody fragments have been expressed on the surface of *E. coli* using the PAL system[20] and the Lpp–OmpA system[21] (Table 1). A single-chain Fv fragment was recently surface-displayed on *S. carnosus* with retained capacity to bind its antigen (human IgE)[22] (Table 2).

A possible major advantage of the bacterial-display systems over the phage-display techniques lies in the fact that bacterial selection might be accomplished through FACS technology, using FITC-labelled antigens. This would avoid crucial steps in phage-display selection procedures such as immobilization of the antigen, elution of bound phages and reinfection with eluted phages. Filamentous phages are too small to be compatible with current FACS technology[6]. It has, however, been shown that *E. coli* cells expressing functional cell-surface-anchored antibody fragments can be separated by FACS[6,21,66]. Francisco *et al.* performed a key experiment for indicating the feasibility of FACS selection: they mixed control and positive cells at a ratio of 100 000:1 and enriched the antibody-expressing cells to greater than 79% of the final mixture in only three rounds of sorting[6,21].

These results, showing that (1) large protein libraries can be surface displayed on bacteria, (2) scFv fragments can be functionally expressed on bacterial cells, and (3) such cells can be efficiently enriched by FACS technology, should increase optimism for the future use of bacterial display as an alternative or at least a complement to the phage systems.

Environmental applications

A very different application of bacterial surface display was recently suggested by Sousa *et al.*[16], who displayed polyhistidyl peptides on the surface of *E. coli* using the LamB system (Table 1). *E. coli* cells with surface-exposed histidine tags adsorbed approximately 11 times more Cd^{2+} ions than control cells. Such bacteria could perhaps be used for bioadsorption of heavy metal ions, potentially valuable for environmental applications[16].

Concluding remarks and future perspectives

A multitude of heterologous proteins have been targeted and anchored to the cell surfaces of Gram-negative or Gram-positive bacteria, and a number of different application areas have been identified. From a practical point of view, Gram-positive bacteria have certain properties that make them potentially more suitable for bacterial surface-display applications[41]. Firstly, the surface proteins of Gram-positive bacteria seem to be more permissive of the insertion of extended sequences from foreign proteins than the Gram-negative surface proteins[7,22,40,45,54]. For Gram-positive bacteria, most systems for surface display rely on a common mechanism for surface anchoring that allows insertion of heterologous protein regions of several hundred amino acids[7,22,40,45,54]. In contrast, Gram-negative outer membrane proteins, which are surface anchored via multiple passages through the outer membrane, have only the surface loops as 'permissive sites' for the insertion of foreign sequences and normally allow only much shorter insertions (one exception was the demonstration that a 232 amino acid sequence of SpA could be surface exposed on *E. coli* using a permissive site in LamB; Ref. 33). A second, more obvious, advantage with the Gram-positive systems is that translocation through only a single membrane is required to achieve proper surface exposure of the heterologous polypeptide. In the Gram-negative systems both translocation through the cytoplasmic membrane and correct integration into the outer membrane are required for surface display (Fig. 2). Finally, considering the practical handling of the bacteria, Gram-positive bacteria have the additional advantage of being more rigid, due to the thicker cell wall[22] (Fig. 3). However, one potential drawback to the use of Gram-positive bacteria is their lower frequency of transformation compared to Gram-negative bacteria, a factor that is of obvious importance in the creation of large libraries. Nevertheless, transformation frequencies of 10^5 to 10^6 transformants $(\mu g\ DNA)^{-1}$ have been reported for *S. carnosus*[47], which indicates that surface-displayed protein libraries of a significant size could be produced in Gram-positive bacteria. Certain Gram-positive

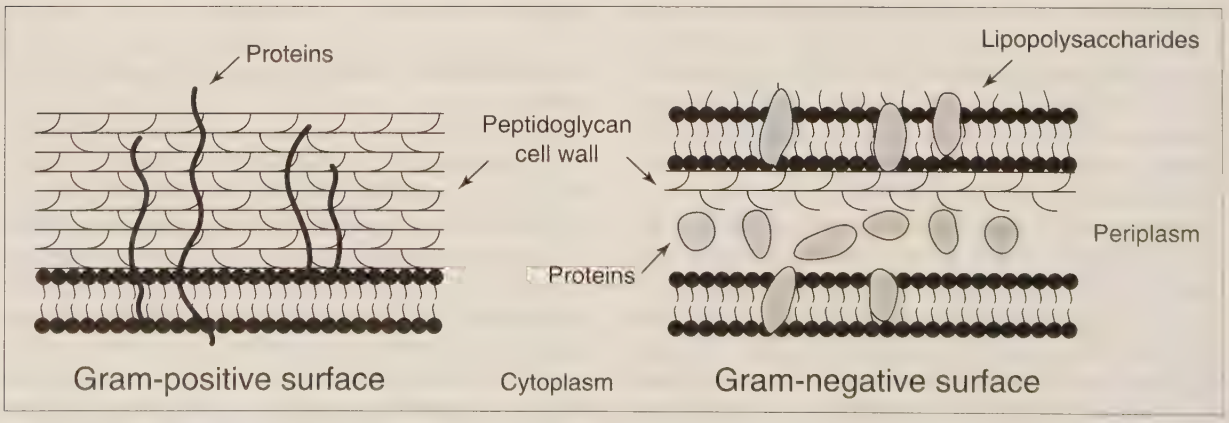

Figure 2
Schematic representation of the differences in the cell surfaces of Gram-positive and Gram-negative bacteria.

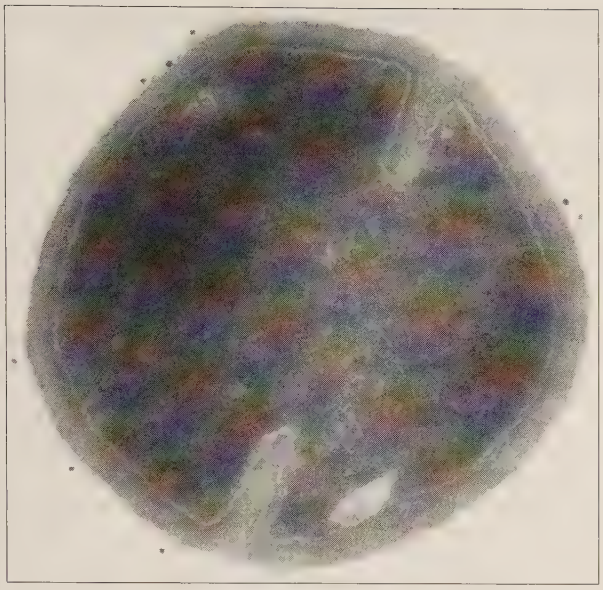

Figure 3
Immunogold electron microscopic picture of an *S. xylosus* cell exposing a streptococcal protein on its surface. The binding of rabbit antiserum specific for the streptococcal protein is detected by the presence of 10 nm diameter colloidal gold particles (*S. aureus*-protein-A–gold conjugate). Note that the cell wall constitutes approximately 10% of the cell radius, which suggests that chimeric surface proteins need to be anchored to the cell wall, and not in the membrane (as for Gram-negative bacteria), to be accessible on the outer cell surface.

bacteria, such as *B. subtilis*, are known to excrete large amounts of proteases, which could cause problems for surface-display applications. In contrast, *S. carnosus* is described as having very low extracellular proteolytic activity[47]. One advantage of the Gram-negative bacteria such as *E. coli* and *Salmonella* is the large number of characterized strains available, which for certain applications have been shown to be useful in the control of surface display[30].

The surface display of active antibody fragments on bacteria could result in a number of new applications. One practical use of this type of recombinant bacteria would be as 'whole-cell monoclonal antibodies' in different diagnostic tests, representing a straightforward and cost-effective way of producing monoclonal antibodies for diagnostic purposes. As a single cell can potentially be detected, it is possible to envisage highly sensitive antibody-based assays. Furthermore, with the help of combinatorial chemistry (such as phage- or bacterial-display technology), there is the possibility of selecting recombinant antibodies or artificial binders with any desired specificity. One interesting application would be the use of combinatorial libraries based on protein domains from bacterial surface proteins (for example, that described by Nord *et al.*[67] utilizing a domain from SpA as a scaffold, which should thus be suitable for display on staphylococcal surfaces).

This concept might also find applications within the field of biofilter development. Ligands selected from combinatorial libraries as specific for a certain compound could be surface displayed on bacteria, which could then be prepared in biofilter form for specific capture. Although enhanced metalloadsorption has been achieved by the surface display of polyhistidyl peptides on *E. coli*[16], the applicability of bacterial biofilter technology remains to be proved.

In the context of vaccine development based on recombinant bacteria carrying heterologous antigenic determinants, the surface display of antibody fragments might become of value. The possibility of expressing recombinant or even 'artificial' antibodies in a functional form on the surface of staphylococci could potentially be used for targeting recombinant bacteria to desired immunoreactive sites. For example, a surface-displayed antibody fragment that binds a certain surface protein or carbohydrate known to be present on an immunoreactive site, such as M cells, could be a means of directing the recombinant bacteria to the desired area and thereby potentially increasing the immune responses to various co-expressed surface antigens.

Among the different bacterial species investigated for vaccine delivery, it is difficult to speculate which types will have the best potential for eliciting protective immunity while being completely safe to use. Food-fermenting bacteria, such as the staphylococcal strains and various kinds of lactic acid bacteria described, as well as commensal bacteria, should be safe to administer orally but may suffer from insufficient immunogenicity. However, the risk of reversion to a virulent phenotype and the potential side-effects in immunocompromised individuals and infants might raise concern over the use of *Salmonella*- or BCG-based recombinant vaccines in humans[68]. One interesting aspect of the choice of bacteria to be used as vaccine-delivery systems is their capacity to withstand the harsh conditions that can be expected during vaccine storage and transportation. In this context, *Bacillus* spores, which are extremely resistant to both heat and cold and which have only recently been considered for vaccine delivery applications[46], may attract future interest.

In conclusion, it is evident that bacterial surface display will be a continuously growing research area in applied microbiology, vaccinology and biotechnology. Both Gram-negative and Gram-positive bacteria of various kinds will be extensively investigated for different biotechnological applications in the near future.

Acknowledgements
We thank Friedrich Götz, Marco Oggioni, Gianni Pozzi, Jerry Wells, Jean-Christophe Piard, Vincent Fischetti and David Acheson for valuable information; Tomas Bächi for preparing the immunogold electron microscopy picture; Elin Gunneriusson for help with the figures; and Marianne Hansson, Sissela Liljeqvist and Fredrik Viklund for assistance with literature searches.

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Inspirational biotechnology

Improving Nature? The Science and Ethics of Genetic Engineering

by M. J. Reiss and R. Straughan, Cambridge University Press, 1996. UK£16.95 hbk (288 pages) ISBN 0 521 45441 7

It might be supposed that a book verging on 300 pages of text on the molecular basis of, and ethical controversies surrounding, genetic engineering would be fairly heavy going. Indeed, this preconception is fuelled by the fact that the book in question is adorned with a very dull and unappealing cover. A quick flick through reveals only a handful of black and white line drawings, which similarly fail to capture the imagination. However, make the effort to get into the text and you are very soon rewarded by some of the most compelling literature on this topic that I have ever read. Indeed, I make no apology at all for using the kind of descriptions normally associated with book reviews in the popular press – I really 'couldn't put it down', and I really did find it 'quite inspirational'.

Before I wax lyrical about the merits of this work, I should get my one reservation out of the way. The introduction claims that the book is written in such a way as to be accessible to the non-biologist. I have to say that this is obviously stated from a biologist's perspective; whilst the bulk of the text would, I am sure, be comprehensible to a non-biologist, as the authors have made admirable efforts to keep jargon and technical terms to a minimum, I remain wholly unconvinced that the introductory section (which covers the basics of genetic engineering) would be fully understandable to a person lacking any formal training in biology. I have personal experience of having to convey biological concepts to non-biologists and am convinced that the material needed to be 'shifted down' in its pitch to reach a broader audience. However, as far as the readership of this journal is concerned, the ethical aspects covered by the book will be of more interest and this coverage takes up the bulk of the text. I can vouch that the ethics discussions are truly accessible to the non-ethicist, with the jargon of this discipline being fully explained and presented in clearly comprehensible language.

Issues are considered in sections relating to the ethical concerns associated with genetic modification of microorganisms, plants, animals and finally humans. In each section, case studies are presented and the ethical dilemmas associated with each are discussed in an informative, enlightening and yet non-directive manner, with a frequent touch of humour and an equally liberal dose of common sense and humanity.

I was so impressed by the breadth of coverage of the topic and the sensitivity and thoughtfulness with which the ethical issues were considered that I shall make this text essential reading for this university's biotechnology degree students, and will recommend the purchase of several copies for the library. I wholeheartedly endorse the book for anyone involved in, studying or just interested in genetic engineering. It is educational, informative and stimulating, but above all it is a very good read.

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Genomics for all?

Nonmammalian Genomic Analysis

edited by B. Birren and E. Lai, Academic Press, 1996. US$39.95 (353 pages) ISBN 0 12 101285 9

Nonmammalian Genomic Analysis presents a collection of protocols dealing mainly with physical mapping (long-range restriction mapping and assembly of arrays of overlapping clones, or 'contigs') of smaller genomes. Such mapping work is crucial for the development of an understanding of genome organization and facilitates map-based gene cloning. If you want to work with genomes of 100 Mb or larger, don't expect to find too much to interest you here! There are no chapters dealing specifically with *Caenorhabditis elegans* or *Arabidopsis thaliana*, the genomes of which are presently the subjects of intensive investigation. Nor is there much discussion of the techniques that are so valuable for their mapping, such as contig assembly by fingerprinting and chromosome walking in yeast artificial chromosome (YAC) libraries. That said, the simple, clear presentation of background material, experimental design principles and protocols for the analysis of smaller genomes should make this a popular work.

The book begins with an introduction of pulsed-field gel electrophoresis, including the basic principles of sample preparation and handling. A well-written chapter extends this theme with a description of electrophoretic karyotyping of fungi, including an extensive list of references to work involving various species. This is followed by a short chapter dealing with the isolation of high molecular weight plant DNA from protoplasts. [No reference is made to the preparation of DNA from embedded nuclei, e.g. Zhang *et al.* (1995) *Plant J.* 7, 175–184, which is probably more suitable for most applications.]

Chapter 4 is an excellent summary of molecular marker systems used in plants (and beyond) but such a long and detailed discussion of genetic mapping seems rather incongruous in what is otherwise a physical mapping text. The next two chapters present the principles behind genome mapping using linking clones, in the context of protozoan parasite genomes, and long-range restriction mapping of bacterial genomes.

The remaining chapters present protocols for the construction of various types of clone libraries and for the assembly of contig maps. Chapter 7 describes the construction of cosmid libraries from bacterial DNA and the construction of contig maps by comparing restriction maps and by chromosome walking. The construction of P1 artificial chromosome libraries of lower eukaryotes is described in Chapter 8. Chapter 9 describes the use of gel-purified chromosomal DNA to identify chromosome-specific sublibraries of African trypanosome DNA. YAC library construction and contig assembly using random genomic probes are discussed in the following chapter, along with methods for the analysis of the *Dictyostelium* genome. The integration of DNA constructs into the genomes of prokaryotes and lower eukaryotes is a vital tool for genome analysis in these organisms. The constructs are often used to integrate enzymatic cleavage sites into more or less well-defined genomic locations. An example of one application of this approach is described. The final chapter deals with contig assembly using iterative hybridization to arrayed libraries of various clone types. The approach is thoroughly described and can be applied to smaller genomes without the need for extensive automation.

To conclude, this is an excellent text for those with little experience of genomics who find themselves embarking upon a project that involves physical mapping within a sub-100 Mb genome.

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When size is *important*

Methods in Molecular Biology, Volume 61: Protein and Peptide Analysis by Mass Spectrometry

edited by J. R. Chapman, Humana Press, 1996. US$69.50 (x + 350 pages)
ISBN 0 89603 345 7

The *Methods in Molecular Biology* series covers a wide range of technique areas in molecular biology [recent volumes include: *Protein Purification Protocols* (Vol. 59) and *Protein NMR Protocols* (Vol. 60)]. The series is distinguished by a stepwise presentation of specific techniques and an extensive and informative 'Notes' section where authors may transmit some of their hard-earned experience to the uninitiated about to embark on a technique. As such, these books tend to be especially useful to graduate students and postdoctoral researchers whilst also providing an authoritative overview of a particular area for more senior researchers. This volume is a valuable and timely addition to the series and provides a readable and comprehensive overview of the analysis of peptides and proteins by mass spectrometry (MS).

The opening chapter, by Peter Roepstorff, emphasizes the contribution of the late Michael Barker (to whom this volume is dedicated) to the field. This chapter is very well written and especially helps to orientate the reader who may perhaps be more familiar with small-molecule MS. The historical development of the various MS techniques, from the electron-impact method requiring volatile derivatives in the 1960s to the matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) methods so widely used today, is traced in a clear and eminently readable way. The second chapter, by the editor John Chapman, provides an overview of the instrumentation available for ionization and mass analysis and summarizes the advantages and limitations of each. Again, this chapter is very readable and comprehensive, providing a useful point of reference for the more specialized protocol chapters that follow.

MS techniques are appropriate for the study of peptides, proteins and other biomolecules because they provide both highly accurate mass measurements and structural data, arising from fragmentation patterns. The 18 protocol chapters provide detailed descriptions of how the following structural studies, among others, may be carried out: amino acid sequencing, peptide mapping, mutation analysis, non-covalent binding (e.g. protein–protein oligomerization), analysis of secondary structure, disulfide bond location, conjugation of anticancer drugs to carrier proteins and post-translational modifications such as glycosylation, palmitoylation and phosphorylation. Other topics described include the derivatization of peptides for sequencing, the effects of detergents on mass spectra, the quantification of neuropeptides and a literature survey of MS studies on haemoglobin. The techniques are clearly explained and cross-referenced to each other. If I have a quibble with the book it is the slightness of the coverage given to the MS analysis of hydrophobic proteins and peptides (a mere four pages) despite their biological importance and the difficulty in obtaining structural data on them.

The book finishes with a number of very informative appendices, which are one of its strongest points. These include extra information relevant to the MS of peptides and proteins, which might be especially useful to a novice in

the field. There is a particularly good description of the effects of the occurrence of different isotopes on the final mass determined and of fragmentation of peptides. These reinforce points made in the protocol chapters but are probably best read at the outset. In addition, there is an extensive and comprehensive listing of some one hundred 1995 publications on the topic.

This book provides a detailed, readable and comprehensive overview of protein/peptide MS and, as such, is essential reading for anyone interested in structure determination in molecular biology.

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Monoclonal evolution

Exquisite Specificity: The Monoclonal Antibody Revolution

by A. Cambrosio and P. Keating, Oxford University Press, 1996. UK£39.50 hbk (264 pages) ISBN 0 19 509741 6

This book provides the reader with a fascinating insight into the development of monoclonal antibodies. The authors portray the development of the technique as a laboratory tool used to help dissect the immune system, and also describe its commercial implementation. They correctly indicate that thoughts of commercial exploitation were not the driving force behind the main research effort. The main characters in the book are naturally the scientists involved in the field in the mid- to late 1970s and the early 1980s. An in-depth discussion of who really discovered monoclonal antibodies is presented to the reader. In addition, the authors review the novelty of the technology with reference to the groups working in similar fields and question how Kohler and Milstein managed to accomplish what others had been striving to achieve. Knowing many of the individuals involved, I think the authors accurately describe the disparate claims and conflicts that arose around the technology. It is refreshing to see this discussed without malice on anyone's part. In fact, I was surprised to find myself somewhat engrossed by the text, with one reservation. I found myself irritated by constant references to either unnamed or fictitious scientists.

The second part of the book concentrates on the commercial implementation of the technology, which was slow to take off. Several companies in the immune assay business disputed the benefits of monoclonals over a high-affinity heteroantiserum. The authors then discuss in some detail the conflicting claims of companies such as Cetus, Hybritech and Ortho.

Little detail relating to the production of monoclonal antibodies is given in the book, which is perhaps not surprising given that the technology is now 20 years old. The authors concede a point well known to researchers in the field, namely that producing monoclonal antibodies is as much of an art as a science. Perhaps one can legitimately question how much the general field of molecular biology is also moving in this direction, with the implementation of commercial kits to complete almost every task the researcher wishes to undertake.

While I found the text easy to read, one thought kept coming back to me: who will buy this book? I cannot see a hard-pressed undergraduate or postgraduate wanting to understand the nuances underlying the discovery of monoclonal antibody technology. Perhaps it will appeal to the scientific historian, but this must represent a very small audience. Having said this, I would recommend it to anyone wishing to undertake a career in science. It gives the reader a fascinating insight into how discoveries are made and the almost inevitable claims and counterclaims that arise as a result of such developments.

The book can perhaps also teach all scientists something about the written word. I wonder if Kohler and Milstein, Koprowski and others ever thought that one day researchers would attempt to dissect the precise meaning of every sentence in their papers. I suspect that, like most of us, they concentrated on describing the science and worried less about the Discussion sections of their papers. Clearly, Cambrosio and Keating have arrived at conclusions that few would dispute, but I wonder if their precise analysis of the literature is really legitimate.

One final thought: in my view, the part that George Kohler played in the development of the monoclonal antibody revolution is understated in the text. His role in this saga must not be forgotten, and it would have been fitting to see a dedication to his memory in the text.

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Recent advances in the microgravity crystallization of biological macromolecules

Workshop

Microgravity experiments concerning the crystallization of biological macromolecules (reviewed in detail in Ref. 1) were initiated in the early to mid-1980s[2–5]. The hope was that crystals of improved quality for X-ray diffraction analyses might be obtained, like those seen in Fig. 1, as a consequence of the elimination of sedimentation and the convective flow inherent in crystallization solutions. Indeed, earlier experiments on conventional crystals, both on the ground and in space, had suggested that this might be the outcome[6–9].

Experiments since then have been dogged by persistent skepticism in a substantial segment of the crystallographic research community[10,11]. Doubt was appropriate and justified for several reasons for example, there was no persuasive explanation of why the elimination of convection and sedimentation should necessarily produce more-ordered or larger macromolecular crystals. Control experiments were difficult to conceive and execute on the ground. There were, at least initially, no accepted quantitative criteria for evaluating relative crystal quality, and many of the early experiments were impossible to reproduce and often poorly documented.

The obvious difficulties of carrying out sensitive, automated experiments in a space-flight environment were compounded by other factors – infrequent flight opportunities, few committed investigators, excessive expectations and overly enthusiastic appraisals based on qualitative data. Chronic technical limitations were imposed by the available instruments all being large in mass and volume, thereby limiting flight access, but at the same time executing relatively few crystallization experiments. While crystallization analyses in conventional laboratories might entail hundreds or even thousands of individual trials, the Cryostat[12] allowed only 14, and the most widely used device, the VDA[13], allowed at most 60 per unit.

Several years ago, investigations of macromolecular crystallization in microgravity split into two paths. The objective of the first was to produce high-quality crystals for biotechnology and research applications, such as X-ray diffraction analysis. The goal of the second line

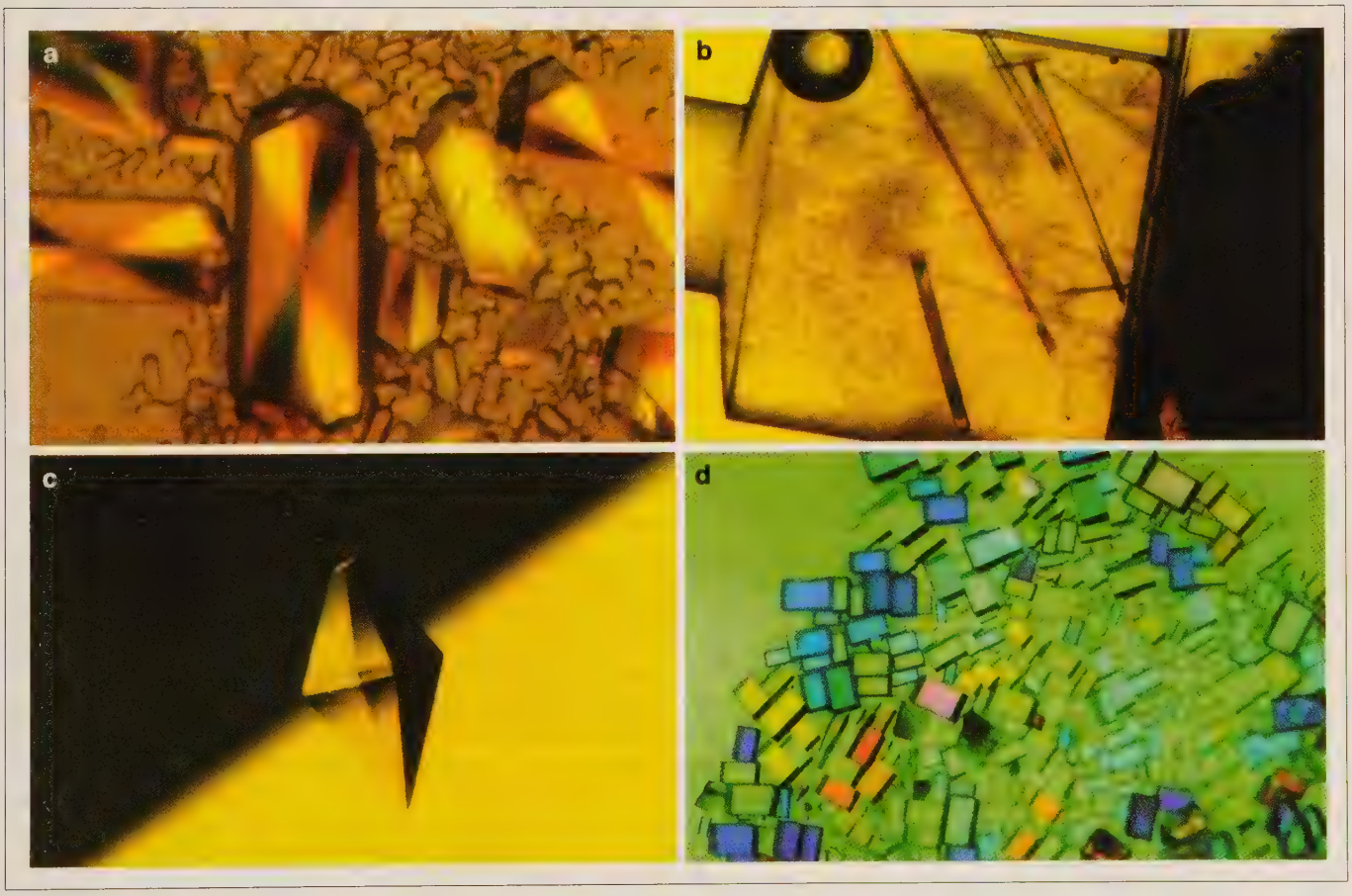

Figure 1

Examples of macromolecular crystals grown in space using four different crystallization devices. (**a**) A large orthorhombic crystal of satellite tobacco mosaic virus grown in the Cryostat[12] on the International Microgravity Laboratory–1. (**b**) Hexagonal crystals of the plant seed protein concanavalin B, nearly 5 mm in length, grown in the hand-held diffusion test cell on the US Microgravity Laboratory–2. (**c**) A single perfect crystal, about 1.5 mm from apex to apex, of the sweet protein thaumatin grown in the European-designed Advanced Protein Crystallization Facility[26]. (**d**) A mass of orthorhombic crystals of the seed storage protein canavalin grown in a Dewar by the flash-frozen, biphasic method on the Russian space station, Mir[29].

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PII: S0167-7799(97)01041-X

of investigation was a definition and description, in a quantitative sense, of the mechanisms by which the quality of crystals was improved in microgravity. Understanding and, in the end, controlling the physics of the process was the objective. This second interest was ably supported by extensive ground-based research using a variety of sophisticated techniques such as static and quasielastic light scattering[14–18], interferometry[19,20], atomic force microscopy[21–25], and X-ray diffraction analyses[5,12,26,27].

The superior quality of space-grown macromolecular crystals

The confluence of results from these two streams has significantly altered the prevailing circumstances and attitudes. Persuasive explanations for the observed improvements in size and quality of macromolecular crystals grown in microgravity have emerged (Fig. 2), and a convincing theoretical framework now exists for understanding the phenomena involved. Physical methods such as interferometry and atomic force microscopy have revealed the unsuspected variety, structure and number of dislocations and defects inherent in macromolecular crystals. These arrays of defects, which provide the key to the improvement attained in microgravity, have been shown to be far more complex, extensive and dense, by several orders of magnitude, than those commonly associated with conventional small-molecule crystals[21,29]. Thus macromolecular crystals are more sensitive to the unusually high degrees of supersaturation at which they are usually grown, and to the mass transport mechanisms responsible for bringing nutrient to their growing surfaces. The self-regulating nature of protein crystallization in microgravity, through the establishment of local concentration gradients of reduced supersaturation, explains why the diffusive transport that predominates in space produces a significant difference in ultimate crystal quality[1].

Another major development is that reliable, quantitative analyses of crystal quality based on X-ray diffraction properties (Fig. 3) have augmented and even replaced earlier subjective criteria for comparing crystals.

The flash-frozen-samples–Dewar approach for multiple crystallizations

Other developments of a more practical nature are also beginning to increase the attractiveness of crystallization in space for the biotechnology community. Serious obstacles to the exploitation of space for commercially oriented macromolecular crystal growth have been the limited

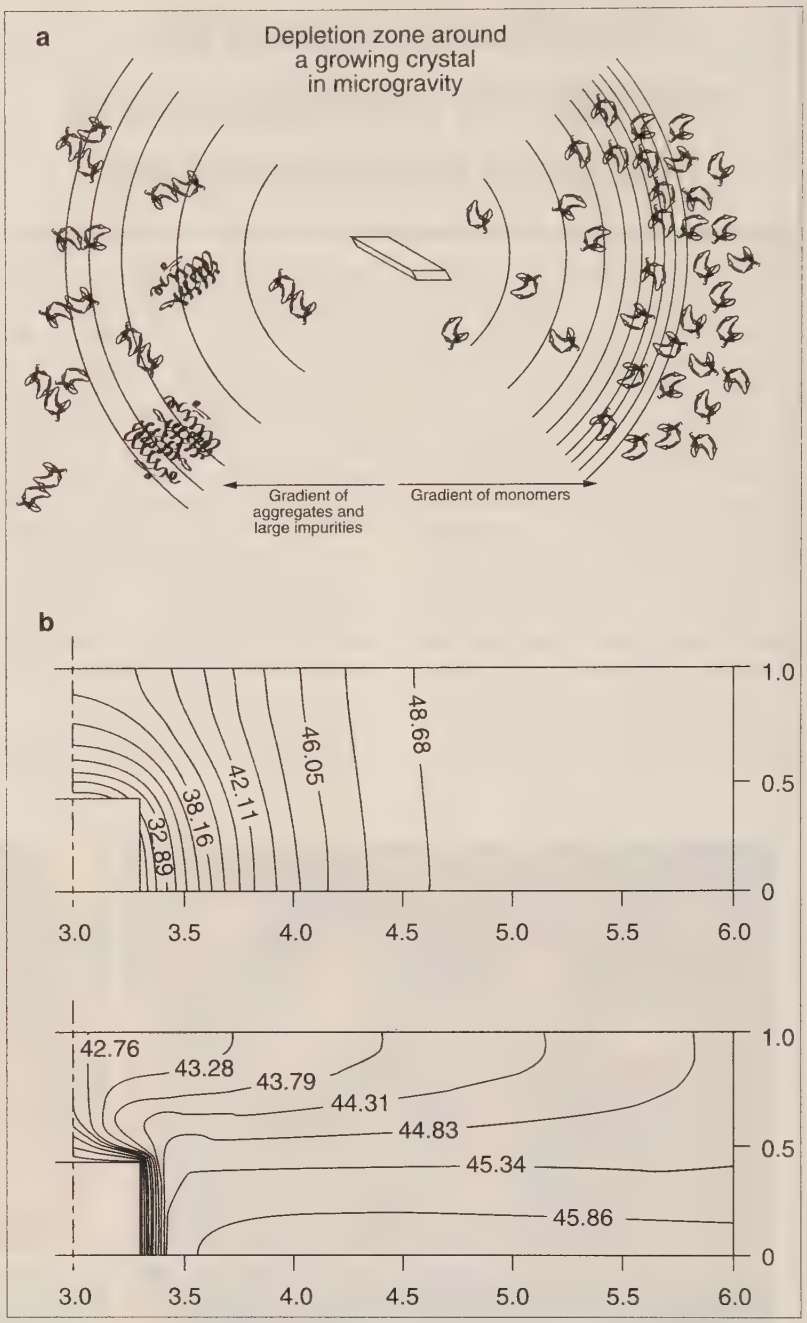

Figure 2

(**a**) The hypothesis underlying the improvement of macromolecular crystal growth in space. As a crystal grows from solution, recruitment of molecules from the surrounding medium produces a concentration gradient that extends from the surfaces. Thus the concentration of nutrient molecules near the crystal is less than that of the bulk solution and the crystal experiences an environment of reduced supersaturation. In microgravity, because there is no convective mixing, the depletion zones are quasistable. The nutrient molecules (on the right) diffuse very slowly because of their large size, thus prolonging and extending the effect. Large impurities, which include other proteins and aggregates of the nutrient molecules (on the left), diffuse even more slowly than monomers; thus the depletion zones serve as a kind of 'diffusion filter' to protect the growing crystal from incorporating some harmful impurities. (**b**) Two mathematical models by Rosenberger and colleagues[28] of the concentration gradients predicted to form around growing crystals in a microgravity environment. The plots show that there can be as much as a 40% reduction in macromolecule concentration between the crystal surfaces and the bulk solution.

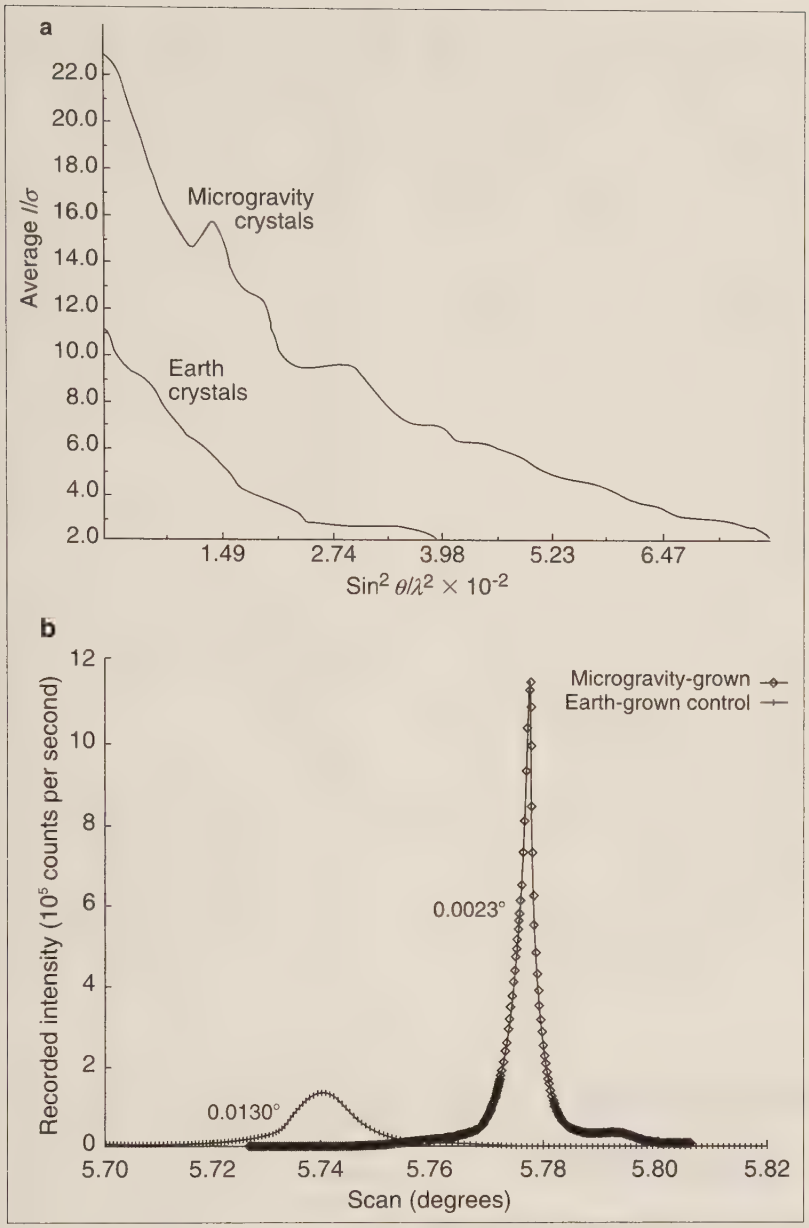

Figure 3

Two types of X-ray diffraction analysis that provide evidence for the superior quality of macromolecular crystals grown in a microgravity environment. (**a**) Intensity/sigma (I/σ) ratios for crystals of satellite tobacco mosaic virus grown in space and in a conventional laboratory on earth[12]. Favorable ratios for the crystals grown in space extend to significantly higher resolution and demonstrate a consistently higher value over the entire range, ultimately providing nearly 50% more X-ray diffraction intensities. Such comparisons have now been obtained for a number of different protein and virus crystals. (**b**) A comparison of the rocking curves for crystals of hen-egg-white lysozyme grown both on earth and in microgravity[27]. The much sharper peak (half-height widths for each peak are given on the graph) for the microgravity-grown crystal clearly indicates a substantial reduction in the mosaic spread, a highly desirable characteristic.

number of samples that could be accommodated on a mission, limitations on sample volumes and, therefore, numbers of crystals, and the expense involved. These impediments have prevented the screening of large matrices of conditions applied to large numbers of proteins to establish growth optima in microgravity. However, a novel approach that largely overcomes many of the problems of scale and economy has recently been described and shown to be successful. This relies on the use of flash-frozen crystallization samples carried into space in a Dewar charged with a small amount of liquid nitrogen.

Details of this methodology, and results of the experiments, have been presented elsewhere[30], but its simplicity, convenience and thrift deserve mention here. Samples of virtually any volume, from a few microliters to several milliliters, can be generated at any time before flight. They are composed of two phases, which were initially liquids – one the macromolecule solution and the other a precipitant solution – that have been sequentially injected or poured into a tygon tube and flash-frozen in liquid nitrogen so that a direct interface exists between the two frozen phases. In this form the inert samples can be stored indefinitely in a freezer until transferred to the flight Dewar and subsequently transported, still frozen, into space. Upon thawing in the microgravity environment, a direct liquid–liquid interface is re-established between the two phases, diffusion of precipitant into the protein solution occurs, and crystallization proceeds by free interface diffusion.

The Dewar has no moving parts, hence no electronic or mechanical overhead, and its entire volume can be used to hold samples. No astronaut intervention is required, and the process is entirely passive. For screening purposes, using 20–40 μl samples in small-bore tygon tubes, about 15 000 different crystallization samples could be carried in the standard NASA Dewar (GN/2) now in use. As described elsewhere[30], the method is amenable to a variety of gradient or multiphase sample configurations in addition to the simple biphasic mode.

The flash-frozen-samples–Dewar experiments have, to this point, been carried out only on the Russian space station, Mir. Dewar units filled with samples are carried to Mir on each space-shuttle–Mir rendezvous mission and exchanged for one from the previous mission. Thus experiments have lasted between 3 and 5 months. In the future, however, Dewars with thermal control will be flown, and these can be employed on shorter (8 to 16 day) US shuttle missions. Dewar-based systems are also likely to be available on the International Space Station beginning in about 2002. In the meantime, the principal investigators invite any interested scientists, from the public or private sector, to provide samples. For at least the immediate future these can be flown at no cost to the co-participants, following toxicological

approval by NASA, whenever flight opportunities arise.

The future

The size of the research community has increased, and with it the diversity of ideas. Opportunities for flight experiments have expanded, along with the variety of instruments with which to conduct crystallization experiments (also reviewed in Ref. 1).

There are currently a number of powerful observational systems under development in the USA, Europe and Japan. These will be deployed on the International Space Station, where they will form the core facilities for the investigation of macromolecular crystallization in space. Those studies will extend and refine our understanding of the physical principles governing microgravity crystal growth and will help identify the properties of macromolecules likely to benefit most from crystallization in microgravity.

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Meeting report

The dawn of the post-genome era, seen from the ocean front

The Human Genome Project is in the sixth of its projected 15 years. The large-scale production of DNA sequence data from human, nematode, microbial and plant genomes is in full swing. New strategies of whole-genome sequencing are emerging, and the challenges and opportunities of the post-genome era are unfolding. The landscape of gene expression and functional studies is being rapidly changed by the newly developed technologies. These are the messages conveyed from the recent, and largest, annual genome-sequencing-community gathering, the Eighth International Genome Sequencing and Analysis Conference, held at the picturesque ocean front of Hilton Head Island, South Carolina, USA during the stormy days of 5–8 October 1996[1]. One indication of the ever-expanding effort and interest is the size of the conference. This year there were over 900 participants, over 50% more than last year. Heavy commercial sponsorship and vendor presence has been one of the distinct features of this conference, illustrating the interest and involvement of industry in genome research.

Production-sequencing progress

After the much-publicized advocation of production sequencing by several key players in the genome community, including Bob Waterston and John Sulston, 1996 was the first year that the Genome Project went into large-scale sequencing-data production. Several genome projects have made significant headway. The *Caenorhabditis elegans* genome project conducted by the Sanger Centre (Hinxton, UK) and the Washington University Genome Center (St. Louis, USA) is still leading in data production, with 58 Mb of the 100 Mb genome sequenced. Nearly 2 Mb of sequence from *Drosophila* chromosome 2 have been completed by the Lawrence Berkeley Genome Center (Berkeley, USA). However, most of these sequenced regions still contain a significant number of gaps, and completion remains a major undertaking.

Certainly, the main thrust is still in the human genome, and there were relevant presentations from a number of groups. The Sanger Centre has been sequencing regions on chromosomes 22 (in collaboration with the Washington University Genome Center), 6 and X (web site: http://www.sanger.ac.uk). The Washington University Genome Center has produced 380 000 expressed sequence tags (ESTs), nearly 200 000 of them unique, and 24 Mb of genomic sequence from chromosomes 22, 7, 5 and X

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PII: S0167-7799(97)01046-9

(http://genome.wustl.edu). The Whitehead–MIT Center for Genome Research (Cambridge, USA) started its human- and mouse-genome-sequencing operation utilizing its own sequence-tagged site (STS) map, with its characteristic massive robotic complexes (http://www-genome.wi.mit.edu). The Institute for Genomic Research (TIGR; Rockville, USA) has undertaken the sequencing of chromosome 16 using the bacterial artificial chromosome (BAC) approach and STS maps (http://www.tigr.org). At the University of Washington Genome Center (Seattle, USA; http://www.genome.washington.edu), Leroy Hood's group is finishing its human and mouse T-cell receptor loci-sequencing endeavor and has started a whole-genome BAC-sequencing program, while Maynard Olson's laboratory has also started to map and sequence chromosomes 7 and 14.

1996 marked the completion of the genome sequence of a eukaryotic model organism, the yeast *Saccharomyces cerevisiae*, as summarized by André Goffefeau (Louvain-la Neuve, Belgium). This was a remarkable undertaking, an international collaboration by a consortium of 96 laboratories from the European Union, Canada, USA and Japan. While the sequence information will give biologists broad avenues of study in years to come, the immediate impact on and applications to gene expression studies have been growing. Ron Davis of Stanford University (Stanford, USA) gave an overview of yeast-whole-genome functional analysis using high-density-array technologies. Peter Philippsen (Basel, Switzerland) introduced the initial effort of the yeast-genome-wide functional analysis by the European Functional Analysis Network (EUROFAN). Similar systematic functional analysis of yeast genes was also initiated by several groups in the USA.

The sequencing of several microbial genomes has also been in full swing. Following the publication of the *Haemophilus influenzae* and *Mycoplasma genitalium* genomes last year, TIGR finished the genome of *Methanococcus jannaschii* this year[2]. The genome of the syphilis-causing *Treponema pallidum* is being sequenced by researchers at TIGR, Johns Hopkins University (Baltimore, USA) and the University of Texas (Houston, USA). Another collaboration, involving TIGR, the Washington University Genome Center and Johns Hopkins University, has been focused on the genome of the pathogen *Helicobacter pylori*. Satoshi Tabata at the Kazusa DNA Research Institute (Chiba, Japan) reported the completion of genome sequencing of the cyanobacterium *Synechocystis*, a photosynthetic organism (http://www.kazusa.or.jp). Over 90% of the coding sequences of plastid genomes of higher plants were conserved in the *Synechocystis* genome, confirming the cyanobacterial ancestry of plastids. The long-awaited *Escherichia coli* project is finally in its closing stages. It is anticipated that an increasing number of genomes of microorganisms of industrial, medical and agricultural importance will be sequenced in the next few years.

Plant genome sequencing has been a rapidly growing field this year, with several collaborative efforts. Over 25 000 *Arabidopsis* ESTs have been generated in US and French laboratories, and the *Arabidopsis* genome is being sequenced in both the USA and Europe. A US consortium including the Stanford DNA Sequencing and Technology Center (Stanford, USA), the University of Pennsylvania (Philadelphia, USA), the Cold Spring Harbor Laboratory (Cold Spring Harbor, USA) and TIGR is taking on the mapping and genomic sequencing of chromosomes 1, 2, 4 and 5. The European Union is conducting the genomic sequencing of chromosomes 4 and 5 as well as continuing EST sequencing. The first comprehensive genome project involving a major crop plant is the rice genome mapping and EST sequencing conducted in Japan, the USA and China, with 30 000 ESTs produced in Japan alone. The agricultural-biotechnology and seeds industries have also started to be proactive in the plant genome field. Pioneer Hi-Bred International (Johnston, USA) cooperated with Human Genome Sciences (Rockville, USA) to generate 46 000 of a projected 200 000 maize ESTs. Other companies including DuPont and Monsanto are following suit.

New tactics and tools

Although a large amount of sequence data is rapidly being produced, it is still a major challenge to obtain large contigs. This to a certain degree reflects the limitation of the traditional approach of cosmid-based mapping and sequencing. With the ever-increasing capacity and efficiency of sequencing techniques, and the ever-increasing production goals and number of finished contigs, a new and robust whole-genome-wide mapping and sequencing strategy has been called for.

Both Leroy Hood and J. Craig Venter (TIGR) advocated a BAC-end sequencing strategy[3]. This would involve the construction of a BAC library with deep coverage of the genome, followed by sequencing both ends of each BAC to build up a minimal sequencing path. The current sequencing techniques and software, such as Phrap by Phil Green (University of Washington, USA), have made BAC sequencing and assembly practical and productive. This approach has been utilized on T-cell-receptor loci by Leroy Hood's group, on the human chromosome 16 project by TIGR, and by groups involved in the *Arabidopsis* genome sequencing project.

The four-color fluorescent chemistry of the ABI sequencer is still the dominant platform for production sequencing. However, a number of emerging technologies have demonstrated promise. The Energy Transfer (ET) dye-primer sequencing reagents by Amersham International (Little Chalfont, UK) have been gaining market share. To compete, PE Applied Biosystems (Foster City, USA) is developing new rhodamine dye-primer and dye-terminator systems. Molecular Dynamics (Sunnyvale, USA) is making slow but steady progress on their 96-channel capillary-electrophoresis sequencer, which would increase sequencing throughput and automation significantly. The Whitehead–MIT group uses a sequencer based on an ultra-thin slab gel to reduce the run time.

There are still a large number of laboratories developing sophisticated robotic systems that aim to integrate several tasks. However, the actual effectiveness of these expensive and unique robotic systems for large-scale production sequencing has yet to be seen. Part of the challenge is to have the development of the automation keep up with the new sequencing strategies, techniques and chemistries.

The prominence of bioinformatics continues to grow quickly, and a considerable number of software and hardware tools have been developed. Recent informatics development

has gone beyond simple sequence comparison and assembly and into more comprehensive database systems that integrate data query, search, link and extrapolation functions. A number of software tools have been developed that incorporate user-friendly interfaces to allow the rapid annotation of sequences by biologists. Several groups addressed these issues. Two presentations by Michael Zhang of the Cold Spring Harbor Laboratory and Steven Salzberg of Johns Hopkins University introduced different methodologies for gene prediction. In addition to the schemes developed in academic laboratories, several commercial packages are available, including GeneWorld by Pangea Systems, Inc. (Alameda, USA) and BioMerge by Molecular Informatics, Inc. (Santa Fe, USA). These should be quite helpful in deciphering the functions of new genes, especially in high-throughput gene-discovery settings.

The post-genome era

With the completion of the genomes of humans and several other organisms in sight, an inevitable question has been posed within the genome community: what will the after-life of the Human Genome Project be? Several presentations at the conference shed light on this post-genome era.

High-throughput and genome-wide gene-expression studies have been directly profiting from genomic sequencing, and in return provide a powerful approach for inferring functional information. The yeast open reading frame microarray has generated much excitement since it was introduced last year. There are currently two approaches to using high-density arrays. One is to spot fragments of amplified cDNA onto the microarray slides, and the other is to synthesize DNA oligo arrays on microchips. Each has its own technical achievements as well as challenges.

The linkage of information about protein accumulation and modification with global gene expression information is another emerging area of research. A group from F. Hoffmann–La Roche (Basel, Switzerland) monitored the global changes in *Haemophilus influenzae* total cellular protein in response to treatment with antibiotics by using a two-dimensional protein gel separation and fingerprinting system coupled with mass spectrometry and database searches. The main limitation of the current system is the sensitivity of the two-dimensional protein separation and detection system.

After 'sequence genomics', 'functional genomics' is next on the agenda. A pressing challenge the yeast research community faces is to identify the function and biological relevance of 6000 yeast open reading frames. Several groups in Europe and the USA are participating in yeast-genome-wide gene-deletion strain production and gene-knockout analysis. A systematic approach for generating single-gene deletion strains using a high-density oligonucleotide array tagging strategy was described by Ron Davis (Stanford, USA).

A unique plant gene trap and enhancer trap transposon tagging system was described by Robert Martienssen of the Cold Spring Harbor Laboratory for studying the function and expression pattern of genes during development in *Arabidopsis*. Similarly, an insertional-mutagenesis-based gene functional analysis system in maize was also reported by Robert Meeley of Pioneer. Gerald Rubin (Berkeley, USA) highlighted *Drosophila* large-scale functional analysis by P-transposable-element insertional mutagenesis and tagging.

Certainly it is no easy undertaking to understand hundreds or thousands of genes whose functions are intertwined and networked. A good starting point would be functional loci. Leroy Hood elegantly illustrated his networked studies on TCR loci from DNA sequencing and phylogenetic comparison to mouse model and immunological studies. Once we start to decipher the genome instead of just individual genes, perhaps we will enter the era of, as Craig Venter put it, 'genomic engineering'.

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by Johannes van den Hombergh and Peter van de Vondervoort

Probiotic properties of lactic-acid bacteria: plenty of scope for fundamental R & D,
by Gerald Tannock

Marine microbial diversity: the tip of the iceberg

Edward F. DeLong

New techniques for evaluating the biological diversity of naturally occurring microbial assemblages combine nucleic-acid-sequencing techniques with molecular-phylogenetic analysis. These molecular techniques avoid many of the selective biases inherent in traditional cultivation-based surveys and provide a universal criterion for identifying and relating diverse microbial species. Application of the molecular approach in marine environments has revealed the existence of unique and previously unrecognized microorganisms. These are providing fresh insight into the ecology, evolution and biotechnological potential of the largely untapped resource represented by the marine microbial world.

Prokaryotic and eukaryotic microorganisms encompass a vast and largely undocumented fraction of existing biological diversity. As prokaryotes have been evolving on Earth for well over 3.5 billion years, the amount of evolutionary diversification within this group is truly immense. In fact, the phylogenetic, biochemical and physiological diversity within microbial groups far outstrips that found within the world of macroorganisms. Microbial diversity can be viewed from a variety of perspectives, including physiological diversity, intraspecific genetic diversity and phylogenetic diversity of species and higher taxa. Obviously, one of the very first tasks necessary for documenting naturally occurring microbial diversity is to assess accurately both the variety and the variability of microorganisms present in any specific habitat. This fundamental goal, so basic for any understanding of the ecology, diversity and biotechnological potential of microorganisms, has rarely if ever been achieved. In the past, advances in the understanding of microbial diversity have been hampered by a lack of appropriate techniques. Only recently has the application of molecular-biological approaches to microbial ecology solved some serious methodological problems. Nucleic-acid-based technologies are changing our perspective on the nature and extent of marine microbial diversity. Recent nucleic-acid-based surveys of marine prokaryotic diversity have revealed unsuspected and novel sources of evolutionary innovation. This evolutionary and biochemical novelty represents valuable grist for the biotechnological mill.

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Marine microbiological diversity: contemporary approaches

Most prokaryotic species can be described as either spheres (cocci), rods (bacilli) or spirals (spirilli), and display few taxonomically informative morphological features, even though some morphological variation in the prokaryotic world is evident (Fig. 1). Consequently, subcellular attributes (physiological, biochemical or molecular) have been the most useful characters for descriptive, taxonomic and identification purposes. However, in many naturally occurring microbial assemblages, it is estimated that less than

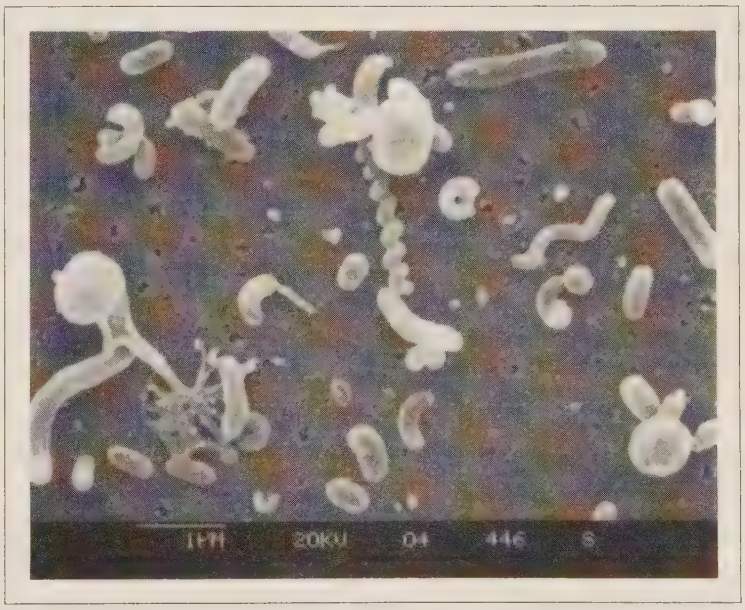

Figure 1
Scanning electron micrograph of marine bacterioplankton. This assemblage was collected in Sargasso Sea surface water. Scale bar, 1 μm.

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PII: S0167-7799(97)01044-5

0.1% of the indigenous microorganisms can be readily recovered by standard cultivation techniques[1–4]. If the majority of microorganisms cannot be cultivated, how can they be identified and characterized and their ecological and biotechnological potential recognized? In the mid-1980s, Pace and co-workers realized that the problem of identifying so-called 'uncultivable' microorganisms could largely be overcome by the application of molecular-phylogenetic techniques[5,6].

By comparing differences in the nucleic acid sequences of homologous genes from different organisms, a molecular genealogy (phylogeny) can be constructed. These analyses provide, in essence, a map of evolutionary history. Organisms close to one another on a phylogenetic tree (Fig. 2) share highly similar inherited traits, and the lines connecting the organisms trace the paths of descent from a common ancestor. Pace and co-workers recognized that phylogenetically informative genes can be isolated, not only from pure cultures of microorganisms, but also from nucleic acids extracted from mixed microbial populations. These genes can then be clonally isolated, sorted and sequenced using standard molecular-biological tools. This sidesteps the need to cultivate individual organisms from the naturally occurring microbial milieu[5,6]. The recovered gene sequences provide a quantitative measure of the identity and evolutionary relatedness of microorganisms present in the original population. Small-subunit-ribosomal-RNA genes were among the first to be used as accurate and reliable molecular-phylogenetic yardsticks, and are still the most empirically useful[7,8]. Reasons for the utility of ribosomal RNA (rRNA) include its universal distribution in cellular organisms, its relatively high intracellular abundance and the large amount of evolutionary information it contains. Comparative analysis of recovered rRNA gene sequences allows rapid phylogenetic identification of naturally occurring microorganisms without cultivation. Recent developments, especially the application of thermostable DNA polymerases and the polymerase chain reaction (PCR) for gene amplification[9–11], have streamlined molecular approaches and made them more accessible to general microbial ecologists.

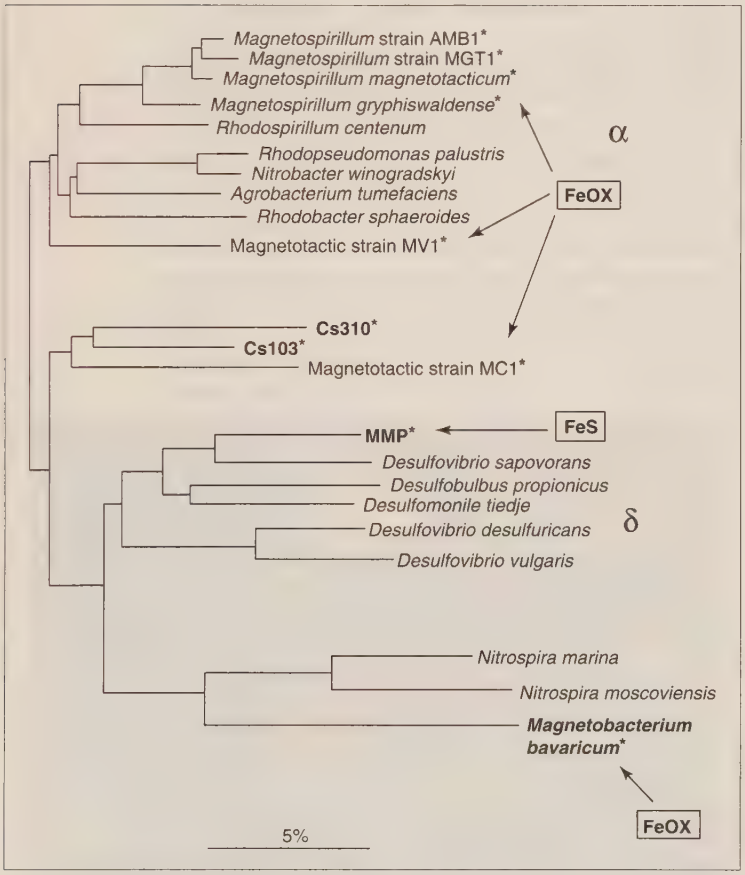

Figure 2

Phylogenetic diversity of some magnetotactic and non-magnetotactic bacteria. An asterisk indicates each magnetotactic bacterium. Uncultivated organisms that have been identified by molecular techniques are indicated in bold. Magnetite-containing organisms are indicated by **FeOX**, greigite-containing organisms by **FeS**. Scale bar indicates five fixed mutations per 100 sequence positions. α, alpha *Proteobacteria*; δ, delta *Proteobacteria*.

The nucleic acid sequence information resulting from molecular-genealogical studies of natural microbial populations yields more than just taxonomic identification. The data provide phylogenetically based markers, or 'signature sequences'[8], which are short nucleotide sequences 15–30 nucleotides in length that are diagnostic for specific taxa[5,6,8,12]. Oligonucleotide probes that base-pair specifically in a Watson–Crick fashion with signature sequences can be designed to serve as nucleic acid hybridization probes. These probes can then be used to detect and quantify the diagnostic signature sequences in nucleic acid extracts or within whole cells, in conjunction with microscopic techniques[4,12]. Again, small subunit rRNAs have proved extremely useful for molecular tagging and identification purposes. Microbial cells typically contain thousands of ribosomes. The high levels of intracellular rRNA provide an abundant target for the phylogenetic identification of individual cells using whole-cell hybridization assays and fluorescently labelled oligonucleotide probes[4,12] ('phylogenetic stains'). Methodological details of nucleic-acid-based phylogenetic approaches in microbial ecology have been well reviewed[4–6]. This article will focus on general themes emerging from the application of such approaches to the study of marine microbial assemblages.

Surveying marine microbial diversity: new vistas revealed by molecular methods

A new and previously unsuspected world of microbial diversity is now being discovered via studies using nucleic-acid-based approaches. Although these studies have been conducted in very diverse microbial habitats, nearly every molecular survey of microbial diversity has arrived at the same general conclusion: *cultivated microorganisms apparently represent only a small fraction of naturally occurring microbial diversity*[11,13–17]. Previously unrecognized microbial groups that are not detected by culture-based surveys sometimes appear to represent dominant species, and form a significant fraction of the total microbial biomass[11,13–17]. Some of

the very first applications of molecular techniques to the study of microbial diversity were conducted in marine environments. A brief look at some of these early efforts reveals some important generalities concerning the extent, nature and potential utility of marine microbial biodiversity.

Gross phenotype may hide underlying evolutionary and biochemical diversity

With very few exceptions, using morphology or other simple microscopic or phenotypic features is a risky approach for identifying and interrelating microorganisms. For example, many biogeochemically important microbial 'types', defined by their conspicuous physiology, actually represent very different microbial taxa that have disparate genetic and phenotypic properties in other respects. Terms like photosynthetic bacteria, nitrifying bacteria (which gain energy via the oxidation of ammonia or nitrite) and sulfate-reducing bacteria (which respire using sulfate as a terminal electron acceptor) all tend to imply close relationship among their component members. Yet these groupings are artificial constructs; each is in reality composed of disparate members, each with its own distinct evolutionary history and biochemical motifs. For instance, sulfate-reducing bacteria are found not only in the delta *Proteobacteria*, but also have representatives in the Gram-positive bacteria and the *Archaea*. Unrelated ammonia-oxidizing nitrifiers are found in both the beta and gamma *Proteobacteria*, and nitrite-oxidizing nitrifiers are found in the alpha *Proteobacteria* as well as two other disparate evolutionary lineages. The diversity contained within these 'functional groups', commonly referred to as single entities, is only beginning to be thoroughly characterized and well understood, largely through the use of molecular-phylogenetic approaches.

Diversity among magnetotactic bacteria

One example of 'hidden diversity' comes from a fascinating group of microorganisms that have evolved the ability to use the Earth's magnetic field to their advantage: the magnetotactic bacteria[18]. These microorganisms contain small magnets, called magnetosomes, which are intracellular, iron-rich, membrane-enclosed magnetic particles. These particles give a permanent magnetic dipole to magnetotactic bacterial cells, orienting them specifically in the Earth's geomagnetic field as they swim. By virtue of their magnetosomes, these microorganisms swim along magnetic field lines to find optimal habitats for their growth and survival[18].

The magnetosomes of most known magnetotactic bacteria contain magnetite (Fig. 2). Phylogenetic analysis shows that many of the cultured magnetite-containing bacteria are associated with a specific bacterial subgroup, the alpha *Proteobacteria*[19–21] (Fig. 2). However, other magnetotactic bacteria collected from sulfidic, brackish-to-marine aquatic habitats were recently found to contain magnetosomes that consist of an iron sulfide mineral called greigite[18,21] (MMP, Fig. 2). The greigite-containing magnetotactic bacteria are not specifically related to other magnetic bacteria, but instead are closely related to sulfate-reducing bacteria within the delta *Proteobacteria*[21]. Additionally, a new magnetite-containing magnetotactic bacterium has recently been described that is not specifically related to any *Proteobacteria*[20] (*Magnetobacterium bavaricum*, Fig. 2). Thus, it can be seen that even magnetotactic bacteria with the same magnetosome mineral-type can be evolutionarily quite different.

These different types of magnetic bacteria can also be expected to vary tremendously in their physiological and biochemical characteristics. Thus the general phenotypic group 'magnetotactic bacteria' contains an impressively diverse array of microorganisms.

Diversity in planetary mixed cultures: planktonic microorganisms of the world's oceans

The term 'picoplankton' refers to planktonic microorganisms that have a cell diameter between 0.2 μm and 2.0 μm (e.g. Sargasso Sea picoplankton, Fig. 1). It was only recently recognized, via direct-fluorescence microscopic counts, that marine planktonic bacteria are extremely abundant, with average cell numbers around 500 000 cells ml^{-1} in ocean surface waters worldwide. However, classical culture-based techniques yield average cell densities (derived from viable counts or colony-forming units) of only about 100–1000 cells ml^{-1} (Ref. 2).

Why do culture-based techniques yield counts that are two to three orders of magnitude less than direct microscopic bacterial counts? One possibility is that the uncultivated microorganisms are the same types that are normally recovered in pure culture, but that in nature they are in a dormant state, making them difficult to recover in pure culture. Alternatively, uncultivated microorganisms may represent previously unrecognized new taxa, incapable of growth on standard microbiological media.

Recent results using nucleic-acid-based phylogenetic techniques indicate that, at least in part, the latter explanation accounts for many ecologically abundant cell types that have never been detected by culture techniques[11,13–17]. In fact, virtually every nucleic-acid-based survey conducted has revealed new groups of previously unrecognized and uncultivated microorganisms[11,13–17]. Sometimes these novel microorganisms are abundant components of natural assemblages and are widely distributed in natural habitats[11,13,14]. A large and growing body of literature indicates that a great deal of microbial diversity has simply been overlooked using traditional culture-based approaches. The very existence of some of these newly discovered microbial groups has challenged accepted theories about evolutionary history and biochemical innovation in the microbial world.

Hidden diversity in the Domain Archaea

An example of one surprise to come from nucleic-acid-based biodiversity studies is the discovery of previously undetected members of the Domain

Archaea. *Archaea* are evolutionarily unique prokaryotes, as genetically distant from *Bacteria* as they are from *Eucarya* (eukaryotes)[7,8,22] (Fig. 3). Cultivated and well-characterized archaeal groups include the extreme thermophiles (which grow optimally at or above 80°C), the extreme halophiles (which grow optimally at sodium chloride concentrations above 2.5 M) and the methanogens (strictly anaerobic methane producers). Because they were previously assumed to thrive only in these 'extremophilic' habitats, the *Archaea* had not been considered very important in aerobic marine environments. Most cultivated hyperthermophiles, for instance (Fig. 3), are restricted to very hot, strictly anaerobic habitats.

Recent molecular-phylogenetic surveys have provided quite a different perspective on the *Archaea*. In cold, aerobic marine habitats, *Archaea* are widely distributed and abundant components of marine plankton[13,14,23,24] (e.g. *Cenarchaeum symbiosum*, 4B7, SBAR5; Fig. 3). Given that *Archaea* were previously thought to be confined to relatively limited and specialized habitats, these findings were quite unexpected. Surprisingly, uncultivated psychrophilic marine *Archaea* have been found to thrive in Antarctic waters at −1.5°C (Ref. 14), yet their closest cultivated relatives are hyperthermophilic (Fig. 3). Several other new archaeal groups have recently been revealed in nucleic-acid-based microbial diversity surveys[15,25]. Uncultivated freshwater mesophilic *Archaea*[25] (Fig. 3) appear to be specifically related to the marine psychrophilic types and are being detected with increasing frequency in a variety of habitats. Another group of uncultivated *Archaea* found in hot springs in Yellowstone Park (JP78 and JP27)[15] appears to be hyperthermophilic and forms the most deeply rooted branch of all *Archaea* in rRNA-based phylogenetic trees (Fig. 3).

The discovery of previously unsuspected archaeal biodiversity has potentially important practical value and application. Currently, hyperthermophilic *Archaea* are being exploited for enzymes and processes that can operate at high temperatures or in the presence of organic solvents[26]. However, the biochemical basis for enhanced protein thermostability is poorly understood. Newly discovered cold-temperature *Archaea* have proteins that are highly similar to those of their hyperthermophilic cousins (E. F. DeLong, C. Schleper and R. Swanson, unpublished), yet these function 0at nearly 100°C lower than their thermophilic

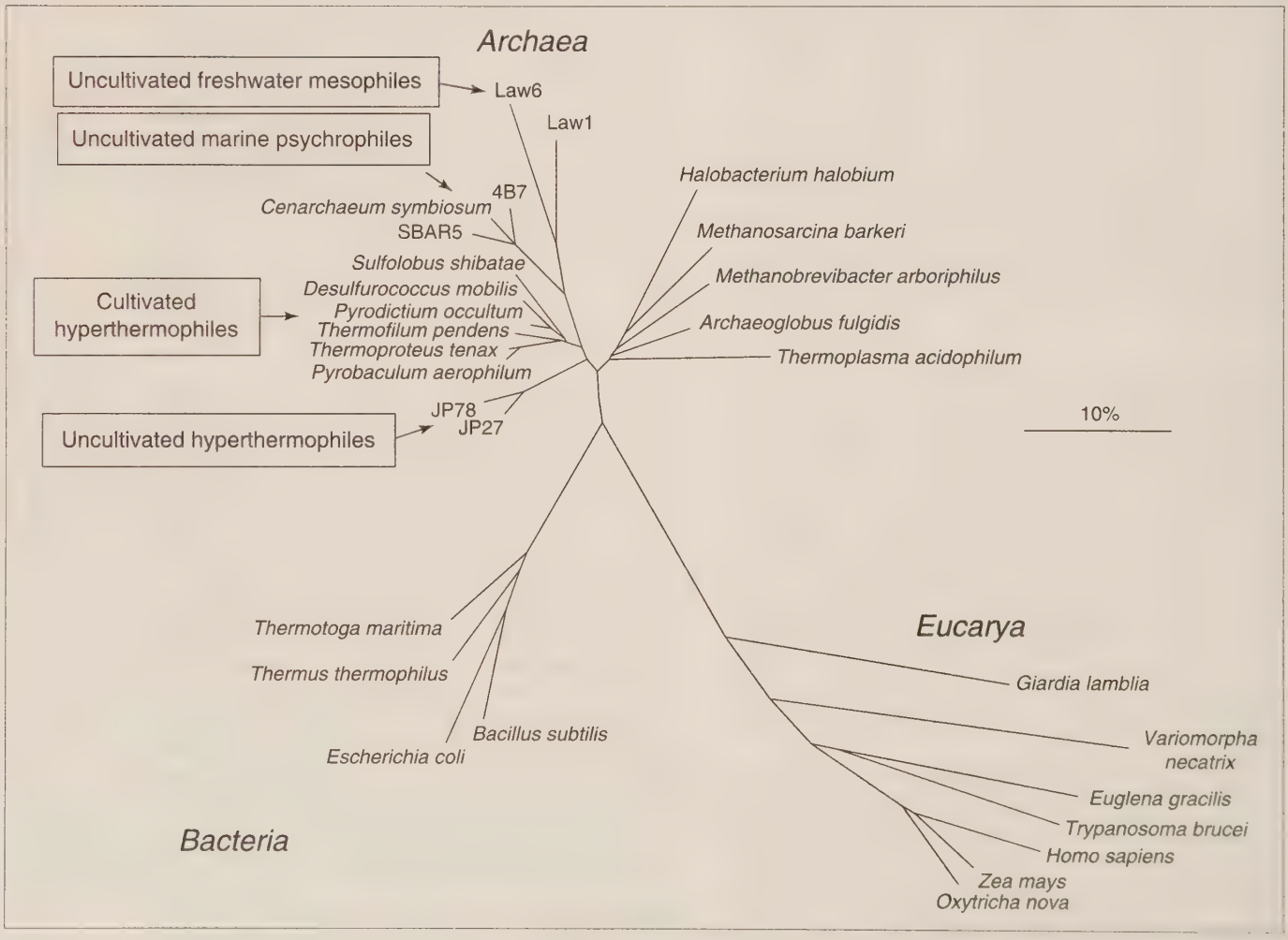

Figure 3

Newly discovered diversity in the Domain *Archaea*. Boxed captions indicate cultured hyperthermophilic *Archaea*, and their newly discovered but as-yet-uncultivated relatives. Scale bar indicates ten fixed mutations per 100 sequence positions.

homologues. Comparative molecular and biochemical studies of newly discovered cold-loving *Archaea* may well contribute important insights into the biochemical basis of protein stability and function at extremely high temperature. Modern genomic analysis is now paving the way for such comparative biochemical and molecular analyses of as-yet-uncultivated cold-loving marine *Archaea*.

Biochemical novelty is a by-product of evolutionary and ecological diversification

Microbial diversity provides a vast resource for novel biochemicals, biosynthetic pathways, enzymes, fermentations and bioremediation processes. However, the biotechnological potential of the microbial world cannot be fully realized until its true phylogenetic and biochemical diversity is well explored, described and understood. For example, the application of thermostable DNA polymerases and the development and improvement of PCR have had a huge impact on molecular-biological and biomedical science. Yet without the pioneering ecological studies and characterization of thermophilic bacterial diversity conducted by Brock in the 1960s (reviewed in Ref. 27), and the subsequent discoveries of hyperthermophilic *Archaea* and *Bacteria*[28], such quantum leaps in molecular technologies would not have been possible. Basic research in microbial ecology[27] paved the way for the revolution initiated by the use of thermostable DNA polymerases in PCR[9].

Summary

The major lesson of recent molecular surveys of microbiological diversity is that a large portion of the microbial world has remained invisible to traditional cultivation approaches. Modern molecular approaches, including genome sequence analysis, now allow access to this previously unrecognized diversity. Genes can now be isolated from uncultivated marine microorganisms, which have never been available in pure cultures, using modern nucleic-acid-based approaches. A good example is the isolation, expression and functional study of ribulose bisphosphate carboxylase from an obligately symbiotic uncultivated marine bacterium[29]. Functional proteins from uncultivated organisms are now being harvested using nucleic acids extracted from mixed populations and uncultivated microorganisms via high-throughput cloning, expression and screening strategies[26,29,30]. Directed approaches in modern genomic analysis are also providing insight into the nature and potential of uncultivated microorganisms. For instance, recent genomic analyses of uncultivated *Archaea* has led to the cloning and expression of a DNA polymerase from newly discovered psychrophilic marine *Archaea* (DeLong, E. F., Schleper, C. and Swanson, R., unpublished). Much more diversity remains to be discovered and described in the marine microbial world. It is difficult to predict precisely what new insights will be derived from new, diverse components of marine bacterial assemblages. It is certain, however, that the new tools available for exploring microbial diversity will continue to yield novel and potentially useful surprises from the marine microbial world.

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In the next issue:

Microbiology of deep-sea hydrothermal vents,
by Daniel Priew

Crystallinity in starch plastics: consequences for material properties

Jeroen J. G. van Soest and Johannes F. G. Vliegenthart

The processing of starches with biodegradable additives has made biodegradable plastics suitable for a number of applications. Starch plastics are partially crystalline as a result of residual crystallinity and the recrystallization of amylose and amylopectin. Such crystallinity is a key determinant of the product's properties. This article describes the influence of processing and storage conditions on starch crystallinity and offers possible explanations for the various properties of starch plastics, in particular for the problems associated with ageing, in terms of the different crystalline structures.

The incorporation of starch into plastics has been spurred by growing interest in enhancing the biodegradability of plastics and thus reducing the amount of persistent plastic waste[1]. Starch is cheap, produced in abundance, biodegradable and available from many renewable sources. Starch plastics can be used for capsules, coatings, garbage bags, flowerpots, loose-fill packaging, agricultural foils and many other disposable items. Although starch plastics are produced commercially, fundamental scientific knowledge about them is limited, especially concerning the relationships between processing, structure and properties[2].

Starch as a particulate filler or destructurized starch in commodity plastics has been used since the 1970s[1–7]. These plastics contained only a limited amount of starch, and were marketed as biodegradable even though only the starch component was degraded. Truly biodegradable starch plastics should contain no non-biodegradable polymers or additives. To obtain these materials [referred to as starch plastics[1,2], thermoplastic starch (TPS) or destructurized starch (DS)[8–11]] granular starch is processed with plasticizers to enable melting and disruption of the semicrystalline starch granule.

There are several constraints on the commercial production of biodegradable starch plastics. First, cost-effective production is likely to require the use of conventional techniques employed for commodity plastics. Second, additives such as plasticizers and melt-flow accelerators, will need to be biodegradable. Third, the properties of the starch plastics will have to be comparable with those of traditional plastics. Starch plastics are sensitive to water and ageing (see Glossary), and are thus not as durable as commodity plastics. Starch crystallizes during ageing as a function of water content. More has to be learned about the structure and crystallinity of starch plastics to allow the design materials with improved properties and increased resistance to ageing.

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Native starch structure

Native starch is the main form of stored energy in plants and occurs as easily isolatable granules. The granules occur in a variety of shapes and dimensions, and are insoluble in cold water and in most organic solvents[12,13]. Starch is a complex homopolymer of α-D-glucose units and is found mainly in two structurally and functionally distinct forms – amylose and amylopectin[13–15]. Amylose is a linear or sparsely branched polymer with a molecular mass up to 10^6 Da (Fig. 1a), whilst amylopectin is multiply and highly branched with a molecular mass of about 10^8 Da (Fig. 1b). Intermediate forms have been reported (e.g. amylomaize), which are polydispersed both in molecular mass and branching frequency[14]. Native starches differ in both water content and the amylose:amylopectin ratio. Most starches, such as wheat, maize and potato starch, contain 20–30% amylose, but starches with an altered amylose content do occur, such as amylomaize (amylose content 50–80%, extracted from maize mutants) and waxy starches (amylose content >99%, extracted from cereal mutants). Starch granules also contain small amounts of noncarbohydrate components such as lipids, proteins and minerals[12].

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PII: S0167-7799(97)01021-4

Glossary

Glass-to-rubber transition – Change of a polymeric material from a brittle, glassy state to a more flexible and rubber-like material.

Glass-transition temperature – Characteristic temperature at which the glass-to-rubber transition of a polymeric state occurs.

Viscoelastic polymers – Polymers having properties of both a liquid and a solid.

Semicrystalline polymers – Polymers with a partially crystalline, ordered structure.

Ageing – Changes over time of the structure and material properties of plastic materials.

Retrogradation – Starch structural changes, such as helix formation and crystallization, during ageing of starch materials above the glass-transition temperature.

Amylomaize starch – Starch extracted from maize mutants particularly rich in amylose.

Waxy starch – Starch particularly rich in amylopectin, extracted from cereal mutants.

Starch has a high degree of supermolecular organization within the granules[16,17], with the amylopectin molecules organized in a radial pattern. Native starch is partially crystalline (semicrystalline), with a crystallinity of 20–45% (Ref. 18). Amylose and the branching points of amylopectin form the amorphous regions in the starch granules. Amylopectin is the main crystalline component in granular starch. The crystalline regions, consisting of double helices of the amylopectin outer chains, are arranged as thin lamellar domains (Fig. 1b)[19]. Additional crystallinity may arise from cocrystallization with amylose and from amylose crystallization into single-helical structures (Fig. 1a), which form complexes with fatty acids or lipids[20,21]. Several types of crystal structures are observed in the granules, denoted V-, A-, B- or C-type, which differ in the packing density of the single or double helices and in water content[22–24] (Fig. 2). The relative abundances of the crystal structures are influenced by the amylose:amylopectin ratio, molecular mass, degree of branching and length of the amylopectin outer chains, and thus by source.

Thermomechanical processing

Several native starches[25–39], isolated from wheat, rice, corn and potatoes, and including waxy maize and amylomaize, have been used in the manufacturing of plastics. In addition, low molecular mass starches and maltodextrins (J. J. G. van Soest and P. M. Kortleve, unpublished) prepared by acid and enzymic hydrolysis have been processed[28,31,32]. Various industrial processing techniques have been used to prepare starch plastics, including kneading, (sheet) extrusion, compression moulding and injection moulding[1,2,8–10,25]. Processing temperatures are in the 100-200°C range.

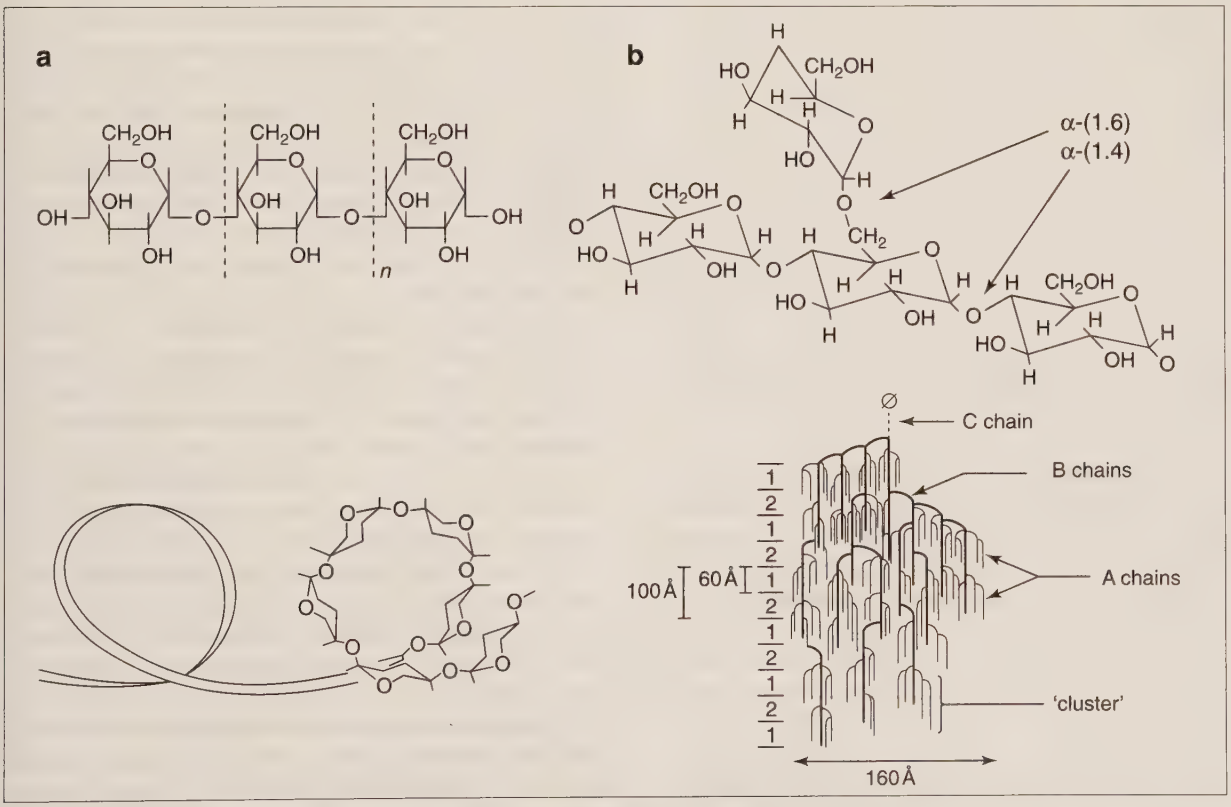

Figure 1

(**a**) Structure of amylose and a schematic representation of an amylose single helix. (**b**) Structure of amylopectin and schematic representation of the crystalline regions (clusters).

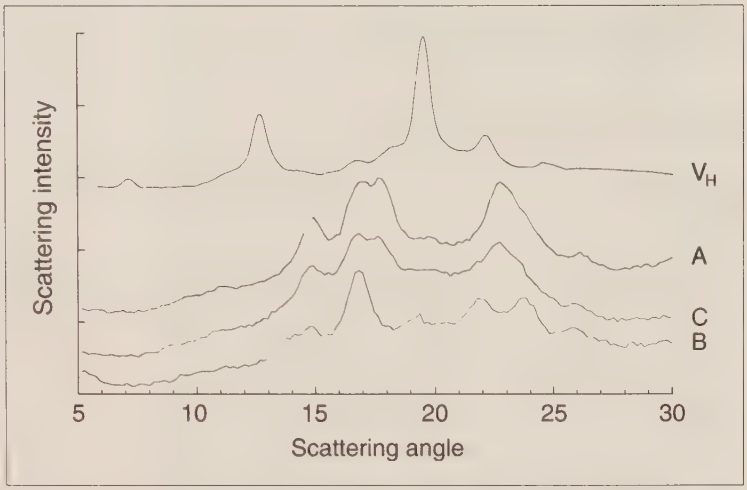

Figure 2
X-Ray diffractograms of maize, potato and pea starch, and crystallized amylose. The peak positions are characteristic of A-, B-, C-, and V_H-type crystallinity, respectively.

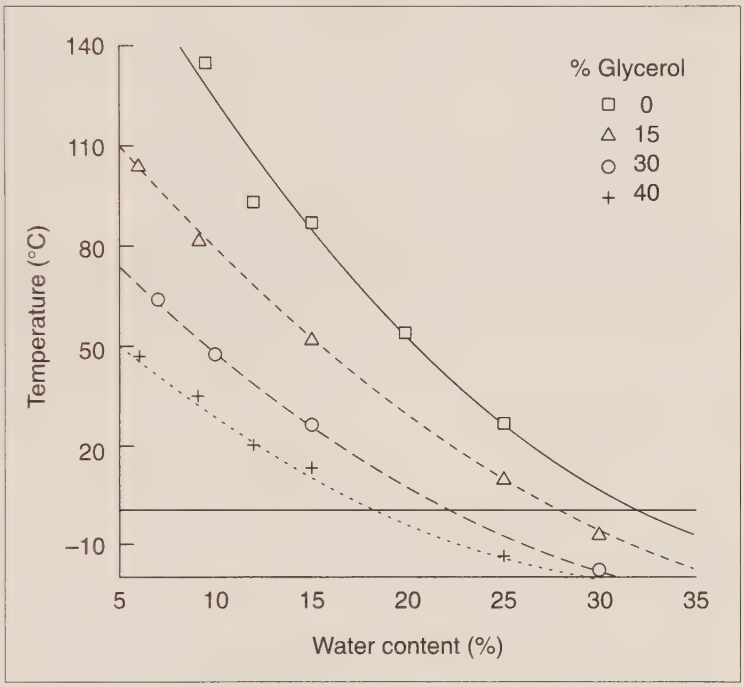

Figure 3
Glass-transition temperature as a function of glycerol and water content.

A variety of shaped products can be obtained, including plant pots, garbage bags and less obvious products such as dog bones and clay pigeons[1]. For analytical purposes, attention has focused on the preparation of sheets and tensile bars. Several plasticizers have been investigated, including water, glycol, glycerol, ureum, poly(ethylene glycol), glucose, maltose and sucrose, as well as melt-flow accelerators, such as lecithin, glycerol monostearate and calcium stearate[2,26].

Polymer-chemical principles of starch plastics

Traditional polymer-chemical principles have been applied to explain the mechanical behaviour of starch plastics[2], which depends on network formation and the starch structures within the network. The network is held together by polymer–polymer and polymer–solute additive interactions, mainly hydrogen bonds and van der Waals forces. Starch materials are viscoelastic and their properties are classified as a function of plasticizer content: a glassy behaviour at low plasticizer content, a rubbery behaviour preceded by a glass-to-rubber transition at higher plasticizer content and a gel-like behaviour at very high plasticizer content. The glass-to-rubber transition is characterized by a glass-transition temperature. Glassy materials are hard and stiff but can be brittle. Materials in the rubbery state behave more as viscous liquids: with increasing plasticizer content the interaction forces between the polymers decrease, and starch-chain mobility increases, which makes the materials flexible and rubbery. The interactions between starch and the plasticizer molecules, especially glycerol, in the rubbery state are still high and the materials are tough. At very high plasticizer content, the materials become soft owing to the low degree of interaction between the starch chains in the amorphous regions, resulting in the plastics behaving more like a gel or paste. The glass temperature of starch-containing materials is a function of plasticizer content[40–42]. An example of the effect of glycerol and water on the glass transition temperature is given in Fig. 3. Polyols such as ethylene glycol, sucrose and fructose are less effective plasticizers than water[2,29,30,34,36,40–42]. Glycerol increases the toughness and strength of the materials because of the strong hydrogen bonding with starch.

Starch structure in starch plastics

Depending on the processing conditions and plasticizer content[2], thermomechanical processing of granular starch with the aid of plasticizers and melt-flow accelerators gives a complex starch, plastic material. This is composed of residual swollen granular starch, partially melted, deformed and disrupted granules, completely molten starch, and recrystallized starch. Starch structure and morphology are determined by the level of disruption and melting of the granular starches and by the ageing processes[28,33,37,38,43–45].

The degree of disruption and melting of the various granular starches is regulated by the plasticizer content (glycerol and water)[33,37,43] and by the processing parameters (shear stress, melt viscosity and temperature)[28,35]. Residual native crystallinity (Fig. 2) is observed in starch plastics, owing to incomplete starch melting. The amount of residual crystallinity is affected by the processing conditions and, indirectly, by the plastic's composition. A continuous network of amorphous starch (DS or TPS) is formed at high temperatures, plasticizer contents and under shear-stress conditions.

Ageing of starch materials in the rubbery state occurs by retrogradation[2,28,29,36–38,44,45], where the starch molecules reassociate in more ordered structures, for example by forming simple juncture points and entanglements, helices and crystal structures. The rate of retrogradation and crystallization is dependent on

the plasticizer content and related to the glass-transition temperature. Crystallization of both amylose and amylopectin occurs.

Processing-induced crystallization of amylose

Processing-induced crystallinity is caused by the rapid recrystallization of single-helical structures of amylose during cooling after processing[28]. Three types of crystallinity have been observed in this situation, and identified as V_H-, V_A- and E_H-type crystals. These crystal structures have characteristic X-ray diffraction patterns, owing to differences in the crystal lattice and hydration. The formation of single helices is mainly determined by the processing parameters, amylose content and additives. During processing, an increase in the input of mechanical energy, which is related to the extrusion screw speed (rate of rotation of the screws) or the processing time during kneading, causes an increase in the amount of single-helix-type crystallinity. The presence of lysophospholipids and complex-forming agents induces amylose crystallization. Complex-forming agents, such as isopropanol, show a strong interaction with starch and are incorporated into the amylose crystals. The amount of crystallized amylose is proportional to the amylose content, and in waxy starch materials, which contain little or no amylose, no single-helix-type crystallinity is observed. The relative amounts of the various amylose single-helical structures and the rate of change are influenced by the temperature and humidity[28]. An example of the E_H-to-V_H transition is shown in Fig. 4.

Crystallization during storage

In glycerol-containing starch plastics, both amylose and amylopectin recrystallize in the B-type crystal structure. At a molecular level, a series of structural changes occurs during storage above the glass-transition temperature. First, double helices and aggregates of double helices (short-range ordering) are formed and then crystallization (long-range ordering) occurs[44,45]. The crystallization rate is influenced by the amylose content, starch source and storage conditions[28,44,45]. Amylose crystallization is fast compared with that of the amylopectin outer chains, which can be either intramolecular or intermolecular[29,30]. Co-crystallization of amylopectin with amylose, which has been suggested for the retrogradation of starch in starch gels[46], probably also occurs[29].

During ageing, the plasticizer content of starch plastics affects the rate of crystallization. Higher amounts of plasticizer cause an increase in the mobility of the starch chains and lower the glass-transition temperature[40–42]. The rate of crystallization increases with increasing water content[2,36–39,44,45]. Conversely, glycerol reduces the crystallization rate at a constant water content[44] owing to starch–glycerol interactions and a reduction in both starch mobility and water stabilization. However, due to the hygroscopicity of glycerol, the water content usually increases, thereby lowering the glass-transition temperature and increasing the crystallization rate.

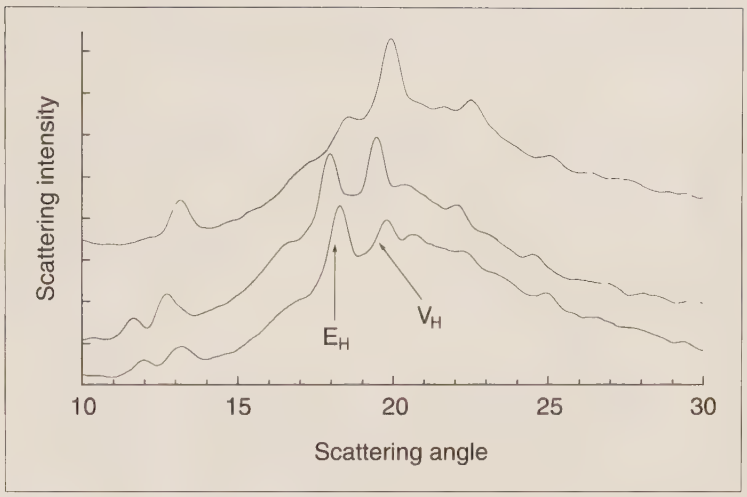

Figure 4

X-Ray diffractometry: the E_H-to-V_H transition (the most characteristic peaks are indicated by the arrows in the figure) due to ageing and water content in starch plastics. The ageing time and water content increase from the bottom line to the top line.

Starch structure in relation to properties

The presence of intact or not-completely-disrupted granular starch leads to inferior mechanical properties, reducing, for example, the stiffness, strength and elongation, as a result of a less-coherent matrix of amorphous amylose and amylopectin[33]. Residual crystallinity within the residual granular starch does not add to the strengthening of the materials[33]. The formation of the amylose network in high-amylose materials leads to a product with high strength and stiffness[37,45]. The linear amylose chains tangle effectively and form strong physical crosslinks, mainly by hydrogen bonding and crystallization, resulting in strong, coherent materials. Amylopectin is effective in increasing the flexibility and elongation of the materials because of its high molecular mass and its highly branched structure[30,33]. The elongation of rubbery materials prepared from partially hydrolysed starches is shown to decrease with decreasing starch molecular mass, because the size of the interaction domains and the long-range coherency (the effective number of chain segments between crosslinks) within the starch matrix is reduced[31,32]. Degradation of starch during thermomechanical processing occurs at high temperatures and shear, and has been shown to affect the mechanical properties of unplasticized starch films[47].

Extruded sheets with variations in plasticizer content, starch source and amylose:amylopectin ratio have been studied. Ageing has been followed directly after extrusion (short-term) and over periods of up to eight months (long-term) at various humidities[48] (Fig. 5). Within the first few days after processing, the material properties of the plastics change rapidly: an increase in the amylose single-helical and B-type crystallinity is observed. The aggregation of the helices and crystallization of amylose are rapid and effective in increasing the strength and stiffness of the network[2,29,36–38]. The observed reduction in plastic flow and elongation is explained by the formation of crystallites that act as permanent crosslinks. At present, the relationship

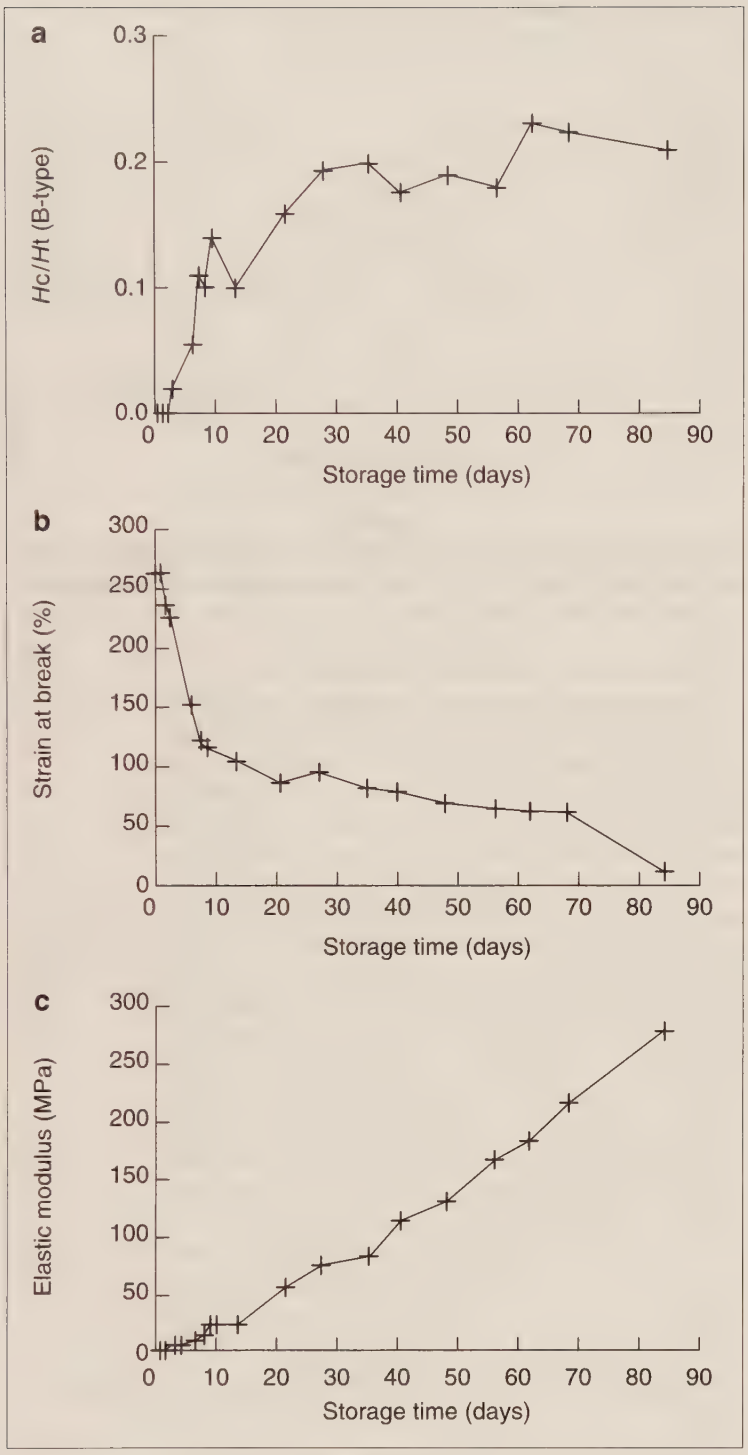

Figure 5

Influence of ageing on B-type crystallinity, strain at break (elongation) and stiffness (elastic modulus) of a starch-plastic material stored at a relative humidity of 54%. The *Hc/Ht* value is a measure of the relative amount of B-type crystallinity.

between the relative amounts of the three types of amylose, single-helical crystallinity and mechanical properties of starch plastics is unknown.

During storage at 60–70% relative humidity for several months or at higher humidities, the materials show a further increase in B-type crystallinity[2,29,30,36]. Although the coherency of the high-amylose materials at 20–35% crystallinity is retained, materials with a high amylopectin content form cracks and shrink at these levels of crystallinity. The effect of crystallization on the properties of amylopectin materials has been explained by the differences in the relative amount of intermolecular and intramolecular crystallization, although the presence of these types of crystallization have yet to be confirmed by structural analysis. Intermolecular crystallization, both amylopectin–amylopectin and amylose–amylopectin, increases the strength and stiffness of the materials because of physical crosslinking. The aggregation and crystallization of helices between adjacent amylopectin molecules is less effective in reinforcing the matrix than the aggregation and crystallization of amylose molecules, because the shorter length of the amylopectin outer chains results in smaller interaction domains. Intramolecular amylopectin crystallization weakens the materials by inducing internal stress and reducing the intermolecular interactions, thus explaining the observed deterioration of starch plastics during storage and use.

Outlook

Although the quality of the present starch plastics has improved, other plasticizers and additives are under investigation to increase their stability and water sensitivity. Increasing our knowledge of the processing events and the relationship between process and structure will lead to an improvement in their properties. However, to increase our understanding of the effects of starch structure on the properties required for specific applications, new and/or improved analytical techniques will have to be developed.

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Applications of microfabrication and micromachining techniques to biotechnology

Chung-Chiun Liu and Zhihong Jin

Silicon-based microfabrication and micromachining techniques have demonstrated their effectiveness in various biotechnology-related applications. In this article, the current utilization of these techniques and the general microfabrication and micromachining processes are discussed. Possible future applications of the techniques to biotechnology are also assessed.

Silicon-based microfabrication and micromachining processes have demonstrated their suitability for producing geometrically well-defined, highly reproducible, microsize, intricate structures for the integrated circuit (IC) industry. In recent years, the application of these processing techniques to the development of microelectromechanical systems (MEMS) has generated much scientific and commercial interest. However, many potential biological applications of microfabrication and micromachining techniques may not be fully realized.

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In biological applications, microfabrication and micromachining techniques have been used to construct microstructures or microsystems that use a very small sample volume (of the order of a microlitre) and that can offer multiple sensing at a modest cost. The techniques have been particularly valuable in the production of microelectrodes and related systems. In recent years, micromachined silicon-based systems have been used for the detection of metabolic activity of living cells[1–6], the investigation of blood cell rheology[7,8] and the recording of the properties of single cells[9,10]. Such studies have substantiated the potential for using microfabrication and micromachining techniques for biological applications.

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PII: S0167-7799(97)01017-2

Present applications

Blood-gas and electrolyte sensors

The use of microfabricated and micromachined electrochemical sensors and ion-selective field-effect transistors (ISFETs) have been reported in both biological and clinical applications[11]. For blood-gas monitoring, dissolved oxygen (p_{O_2}), dissolved carbon dioxide (p_{CO_2}) and pH can be measured with microsize electrochemical sensors. In general, the miniaturized p_{O_2} sensor is based on an amperometric sensor that measures the current produced by oxygen reduction as a function of the oxygen activity. A two- or three-electrode configuration fabricated by silicon-based microfabrication techniques has been proposed[12,13]. The p_{CO_2} sensor is commonly constructed according to Severinghaus' approach[14], which employs a pH sensor and a hydrogen carbonate solution. This pH sensor can be a pH ISFET or a microsize metal–metal-oxide pH sensor[15]. Silicon-based microfabrication techniques have been used to produce both the pH and the pH-based p_{CO_2} sensors[16,17].

Measurements of K^+, Na^+ and Ca^{2+} in biological samples are often made using ion-selective electrodes. The electrode operates in the potentiometric mode, and the ion-selective membrane uses a polymeric matrix in which various ionophores (tridodecylamine or phenoxazine derivatives) can be incorporated. Both the potentiometric electrode structure and the formation of the ion-selective membrane can be accomplished using microfabrication techniques[18]. Obviously, a desirable microsystem would incorporate an array of these sensors for simultaneous measurements of the blood gases and/or electrolytes.

Probe arrays in DNA analysis

The potential development of DNA probes for DNA sequencing using microfabrication and micromachining techniques has generated much interest; in particular, the use of an array of DNA probes for the sequencing of a long stretch of DNA in a single simultaneous process is very attractive. To this end, a probe array has been developed that uses an addressable surface-electrode array to control the attachment of the probes and the hybridization and release of sample DNA[19]. Capillary electrophoresis with microchannels fabricated by micromachining techniques is important for DNA analysis[20,21]. A charge-coupled device (CCD) array of 420 × 420 elements positioned 200 μm apart has also been used for the detection of fluorescence from hydridized DNA[19].

Microphysiometry

Microphysiometry provides an effective method for detecting physiological changes in cultured cells, including the measurement of cellular metabolism, by measuring the rate of extracellular acidification of cells confined in a very small volume. Silicon-based microfabrication technology has been used to produce the microvolume structure as well as the biosensor element. For example, the seminal work by Bousse and colleagues[22–26] used whole cells as the biological component, a silicon-based pH sensor (described as a light addressable potentiometric sensor) and a single sensor chip for a single flow channel. More recently, a single sensor chip with multichannel capability has been used[26]. The integration of fluidic elements will be a logical step for the future advancement of the microphysiometric technology. A channel volume as small as 0.3 μl can be produced by micromachining techniques, and an eight-channel structure on a single silicon substrate (23 × 23 mm) has been reported[26].

The use of microfabrication and micromachining techniques to produce microsize sensors or sensor arrays for biological and medical applications has advantages including better long-term stability and higher packing density[27].

Other biological applications

The use of microelectrode arrays for two-dimensional planar mapping has also been realized. Hinkers *et al.*[28] reported an amperometric microsensor array with 1024 individually addressable elements for two-dimensional concentration mapping. The total surface area with 1024 addressable elements is 1 × 1 cm. A microsize amperometric oxygen sensor in a two-electrode configuration with a platinum cathode and an Ag–AgCl anode is used.

In addition to ultramicroelectrodes and blood-gas and electrolyte sensors, biosensors involving enzymes or other biocatalysts can also be fabricated. Laurell and Rosengren described a micromachined enzyme reactor that can be used for biochemical sensing[29]. The chemical anisotropic etching technique is used to produce an array of deep channels that provide the lamellar structure of the reactor. The enzyme is then immobilized in the reactor. Peroxidase (EC 1.11.7) and glucose oxidase (EC 1.1.3.4) have been immobilized using conventional techniques and the resultant microenzyme reactor used for sensing hydrogen peroxide and glucose[30].

Microfabricated structures and devices have also been used in many other biological and biomedical applications, such as studies of blood cell rheology and cellular flow characteristics[7,8], the study and enhancement of the biocompatibility and adhesion of cells and tissues in orthopedic and dental implants[31–33], and in investigations of fungal growth[34]. Recognizing the effects of surface topographic structures on cell growth, Fuhr *et al.* have proposed an active means of controlling the surface adherence of cells[35]. In this approach, an array of ultramicroelectrodes with a high aspect ratio is microfabricated and subjected to an applied potential. This results in the repulsion of cells at the electrode surface, providing an active biorepellent surface.

Microfabrication and micromachining technology

A significant number of silicon-based microfabrication processes have been derived from the advancement of manufacturing in the IC industry. Of the various processing techniques, lithographic reduction,

thin film metalization, photoresist patterning and chemical etching have found extensive chemical and biological applications, particularly in sensor-related development. However, these processes produce mainly two-dimensional planar structures, which have limited application.

Micromachining technology is generally defined as the means of producing three-dimensional structures, and includes bulk- and surface-micromachining techniques. Chemical-anisotropic and dry etching, the sacrificial-layer method and LIGA (lithographic, galvanoforming, absorming) have all been used in micromachining fabrication. Chemical-anisotropic etching is an etching procedure that depends on the crystalline orientation of the substrate. For silicon etching, potassium hydroxide and tetramethylammonium hydroxide solutions are the most common etching agents. Dry-etching processes include ion milling, plasma etching, reactive-ion etching and reactive-ion-beam etching. These etching processes are not dictated or limited by the crystalline structure; however, although few chemicals are required, the equipment cost of these processes is relatively high.

The sacrificial-layer method employs a deposited underlayer that can be chemically removed. It has been used to create cantilever-type structures for physical sensor and actuator applications. Prohaska[36] used the sacrificial-layer method to make a chamber-electrode structure, which protects the integrity of the sensor element.

LIGA techniques have been used to produce high-aspect-ratio microstructures. These microstructures can be used to define volumes of biological samples, as well as for microanalytical elements such as microcapillary structures.

For many biological applications, it is preferable to have the system operating at a physiologically compatible temperature of approximately 37°C. Incorporation of a heating element and a temperature-sensing element to provide feedback control of the operating temperature has been proposed, and the applications of micromachining techniques to accomplish this goal have been demonstrated[37]. In these microstructures, a small thermal mass is desirable to minimize heat loss and heating energy consumption. This is accomplished by selective removal of the silicon substrate, producing a suspension or diaphragm structure.

Future applications of microfabrication and micromachining techniques to biotechnology

Biosensor applications

The continuing advancement of microfabrication and micromachining techniques will lead to further biological applications. One area of development will be biosensors and biosensor arrays. Many biosensors, including blood-gas sensors, are of an electrochemical nature. Potentiometry-based sensors are commonly employed as sensing elements for biosensors. Such sensors measure the potential established at the interface of the working electrode and the electrolyte, and the potential is not affected by the surface area of the working electrode. However, potentiometric measurement is governed by the Nernst equation, which predicts a semi-logarithmic relationship between the sensor's electrochemical potential and the activity (approximately the concentration) of the analyte. This relationship limits the sensitivity of the sensor.

In contrast, where they are applicable, amperometry-based sensors provide a linear relationship between the sensor output (current) and the activity of the analyte. This is attractive in terms of sensitivity, but the working electrode surface is critical in amperometric measurement and well-defined, highly reproducible electrode surface areas are essential. Microfabrication and micromachining technology make it possible to manufacture a well-defined electrode surface area. Thus amperometric sensing elements may expected to be increasingly used for biosensors.

Voltammetry is another electrochemical sensing principle that requires a well-defined working electrode surface area, and employs a three-electrode configuration operating with a potentiostat. The advancement of solid-state electronics, which has led to the availability of small, portable and modestly priced potentiostats, coupled with microfabricated well-defined working-electrode surface areas will no doubt increase the use of voltammetric sensors in biosensor research.

Microelectrode applications

The use of ultramicroelectrodes and arrays of microelectrodes in biological applications will be enhanced. The incorporation of various biocatalysts and the operation of sensor elements at different selected temperatures for sensor arrays will result in powerful multiple measurements in biological studies. In this respect, methods for the proper dispensation of the biocatalysts on a microstructure will be important. The development of methods to handle the large quantity of data derived from an array comprising a large number of sensing elements or electrodes will also be critical. Artificial intelligence, or other similar techniques to interpret and intelligently use the vast amount of information collected, will be necessary.

Microfabrication and micromachining techniques, including those for MEMS applications, are silicon-based procedures. Consideration of materials other than silicon for potential biological applications will be needed, depending on the application. For instance, the hydrophobic nature of silicon is undesirable when there is any contact with aqueous biological samples. Thus either modification of the silicon or the selection of another, more biocompatible, material must be addressed. The silicon substrate may be modified using the plasma-enhanced-etching technique. Other materials that may be considered for use in microsystems include quartz, polyimide and inert metals such as titanium.

Other biological applications

Despite the current success of microcapillary electrophoresis, further development of this area is

required. The microcapillary structure needs to be integrated with valves, fluidic systems and even pumps. These components have all been fabricated individually by micromachining techniques, but integration on the same substrate has not been accomplished. Such integration may require changes in the microfabrication and micromachining procedures.

Packaging

Packaging of the microfabricated devices is an important and necessary facet. In biological applications, these devices are likely to have direct contact with samples. The long-term stability and sensitivity of the devices often suffer owing to the adsorption of biological components onto the surface of the device or other interactions of the components and the surface. These phenomena will be more prevalent in a microsize device or system. While there is no fixed rule that dictates the approaches to packaging microdevices or microsystems, consideration must be given to the maintenance of the desirable functions of the device as well as to the integrity of the device itself. The use of selective membranes or protective coatings can provide a degree of protection for the system, but long-term stability remains elusive. Thus researchers need to be realistic about the maximum operational life of a microsystem, and to replace it as required. Microfabrication and micromachining techniques can be used to manufacture the microdevices and microsystems at modest cost; consequently, a disposable or replaceable device remains economically and technically viable.

Summary

Silicon-based microfabrication and micromachining technologies have demonstrated their potential in various biological applications. The use of these techniques will increase significantly. However, various issues such as consideration of the materials used, the handling of data, the selection of the operational mode of the system and the packaging of the system will require further research and development, which will be a multidisciplinary effort. This technological challenge is both scientifically and economically significant and provides excitement and inspiration in this important endeavor.

Acknowledgements

We appreciate the support of the Edison Sensor Technology Center, an affiliate center of Cleveland Advanced Manufacturing Program, CAMP, in providing a stimulating environment for biosensor research.

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Biomedical applications of nanotechnology – implications for drug targeting and gene therapy

S. S. Davis

Colloidal particles in the nanometre size range (less than 1 μm in diameter) can be engineered to provide opportunities for the site-specific delivery of drugs after injection into the general circulation or lymphatic systems. Targets include the liver (both Kupffer cells and hepatocytes), endothelial cells, sites of inflammation and lymph nodes. The size and surface of the particle are crucial factors in targeting, and the attachment of cell-specific ligands can lead to increased selectivity. The applications of such particle engineering are discussed in relation to conventional drugs as well as the emerging area of gene therapy.

Within medical practice, there has long been the desire to achieve selective delivery of drugs to specific areas in the body in order to maximize drug action and minimize side effects. It is well known that many drugs, while having a beneficial action, can also exhibit deleterious effects that may limit their clinical utility. Drugs used in cancer chemotherapy represent a clear example of this problem. Cytotoxic compounds can kill target cells, but also normal cells in the body.

The magic-bullet concept, first expounded by Erlich, represents an early description of the drug-targeting paradigm. Such targeting can be achieved with different levels of sophistication: first-order targeting can be defined as the delivery of a drug to a particular organ; second-order targeting is where the drug is directed to a specific cell type; and third-order targeting is where the drug is directed to a structure within a cell. In some therapies first-order targeting will be adequate, whereas in other situations third-order targeting is essential (for example in gene therapy, where targeting to the nucleus is a prerequisite of gene expression)[1].

Targeting systems

Various methods have been proposed for achieving targeted drug delivery. Such methods can be subdivided into chemical, biological and physicochemical strategies. The chemical methods involve the use of modified forms of active drugs known as *prodrugs*. These inactive compounds can accumulate preferentially in target sites and then be cleaved to release an active compound, normally by means of an enzymatic process. For such prodrugs to be effective it is important that there is a clear discrimination between target and nontarget tissue. Some prodrugs work by exploiting differences in pathophysiological conditions (e.g. pH, enzyme content) within target tissues. However, in many cases effective discrimination is not easy to establish[2].

Biological approaches to drug targeting include the use of monoclonal antibodies, where a conjugate of the drug is formed by coupling it chemically to an antibody or a fragment thereof, using an appropriate spacer. The conjugate can then be directed towards an antigen residing on or within the target tissue. Unfortunately, it is difficult to find target tissues bearing specific antigens that will provide a unique targeting effect. For example, for chemotherapy, tumour-specific antigens are rare: tumour-associated antigens can be present not only within the target tissue but also elsewhere in the body. As a consequence, the conjugate may be delivered both to the target site and to nontarget tissues, where toxic side effects can occur[3].

Other biological methods for targeting exploit ligands in the form of sugars or lectins, which can be directed to specific receptors found on cell surfaces. The carbohydrates glucose, mannose, galactose and mannose-6-phosphate are relevant in this regard. Monoclonal antibodies and ligands can be combined with other targeting modalities, especially those based on physicochemical methods, such as polymers and particles.

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PII: S0167-7799(97)01036-6

Polymers and particles

The use of physical methods for the site-specific delivery of drugs in the form of polymeric and colloidal carriers has attracted much attention[4]. Here targeting can be achieved either by 'passive' methods, where a polymer conjugate or particle system is captured by a physiological uptake mechanism such as filtration or macrophage sequestration, or by 'active' targeting, by the attachment of a homing moiety such as a monoclonal antibody or ligand, as discussed above. Polymer–drug conjugates directed towards liver tumours have been well described[5]. Equally popular have been particulate systems in the form of microparticles (nanoparticles), liposomes and emulsions. This article will focus upon such particulate carriers and will consider their new-found relevance in the emerging field of gene therapy.

Nanoparticles and drug delivery

The term nanoparticle is used in this article to cover particulate carrier systems (intended for drug delivery) that are less than 1 μm in size and normally below 500 nm. These particulates can include not only solid structures in the form of nanocapsules but also small liposomes and emulsions. Nanoparticles can display interesting properties *in vivo*, and in some cases can escape from body compartments to defined tissue and cell sites. There are instances where such particles can also reach intracellular structures. Nanoparticles can be given by a variety of routes, depending on the desired therapeutic outcome. They can be used not only for the delivery of drugs but also for vaccine administration and for diagnostic imaging[6]. In this article, the vascular and lymphatic compartments of the body are selected as target 'tissues' to illustrate the potential of nanoparticles for drug targeting, with a special emphasis on gene therapy. Nanoparticles can also be administered to other sites in the body, for example the gastrointestinal tract (for the induction of mucosal immunity), into the lungs (for the treatment of asthma) or into tissues or compartments by parenteral dosing (e.g. subcutaneous, intramuscular, intrathecal and intra-articular injection).

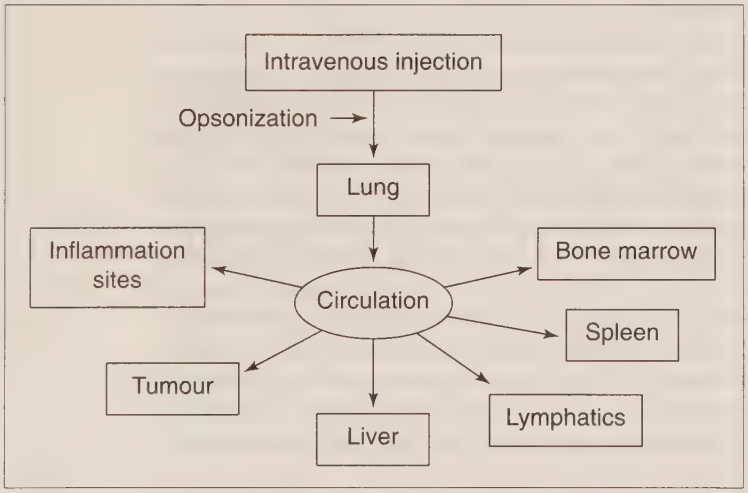

Figure 1

The fate of a nanoparticle following intravenous (i.v.) injection. The crucial stages that can influence the organ uptake of an injected nanoparticle are shown. Particles are conditioned immediately on injection by plasma proteins (opsonization). Large particles are trapped in the liver, while smaller particles can reach the general circulation. Particles that are recognized as foreign are removed by the liver but particles with suitably modified surfaces can remain in the blood circulation or be 'directed' to sites of inflammation, endothelial cells or the spleen.

Nanoparticle fate after intravenous administration

The established fate of nanoparticles following intravenous administration indicates the diverse physicochemical and biological events that need to be considered in the design of a colloidal targeting system. After the particles are injected into the blood stream (Fig. 1) they are rapidly conditioned (or coated) by elements of the circulation, such as plasma proteins and glycoproteins. This process, known as opsonization, is critical in dictating the subsequent fate of the administered particles. Normally, for nanoparticles (and other foreign materials that are recognized as foreign) the opsonization process renders the injected material easily recognizable by the major defence system of the body, the reticuloendothelial system or, more correctly, the mononuclear phagocyte system. The macrophage (Kupffer) cells of the liver (and to a lesser extent the macrophages of the spleen and circulating macrophages) play a critical role in removing opsonized particles[7]. Kupffer cells are known to possess a wide range of different receptors that can be triggered by a coated foreign particle; receptors for the Fc portion of antibodies, for elements of the complement cascade and for fibronectin all reside on Kupffer cells. The process of particle capture and recognition is normally extremely efficient and can be modulated by particle size and, to an even greater extent, the surface properties of the particle. Particles that have a largely hydrophobic surface are efficiently coated with plasma components and rapidly removed from the circula-tion. However, particles that are more hydrophilic can resist this coating process to a variable extent and, consequently, are cleared more slowly (see below)[8].

Passive targeting of nanoparticles

Uptake of nanoparticles by the Kupffer cells of the liver represents an excellent example of passive targeting. In certain limited cases, this targeting can be exploited to help treatment. For example, in some disease conditions, such as leishmaniasis, listeria and candidiasis, macrophages are directly involved in the disease process[9].

In addition, the capture and degradation of particles by macrophages has been exploited to achieve the controlled delivery of drugs to the blood circulation. Macrophages sequester particles through the process of phagocytosis: a particle is engulfed and presented to an endosomal compartment and thence to a lysosomal compartment. The lysosome contains a vast array of enzymes that are capable of degrading particles (and in some cases the encapsulated drug, particularly if it is a peptide or protein). An example of controlled drug

delivery via the lysosomal system of macrophages is provided by incorporating the anticancer agent adriamycin into liposomes. The liposomes are captured rapidly and efficiently by the liver following intravenous injection and moved into the lysosomal compartment, where they are degraded. The drug is released and is able to diffuse into the circulation in a controlled manner. This strategy, when applied to solid, slowly degraded liposomes, allows the drug to be delivered gradually to the blood, thereby leading to a reduction in side effects. In contrast, the bolus administration of adriamycin to the circulation can lead to high transient concentrations that cause cardiotoxicity[10]. A similar approach has been adopted for the antifungal agent amphotericin, where the site of toxicity is the kidney (and to a lesser extent the heart) after bolus administration or release[11].

However, in many situations, the Kupffer cells of the liver are an inappropriate target and represent a major barrier to the targeting of particulate carriers to other sites in and beyond the vascular compartment. Therefore, over many years, methods for disguising particles so that they are no longer recognized by liver macrophages have been explored. In the early 1980s, our group, in collaboration with Lisbeth Illum in Denmark, discovered that it was possible to disguise particles by making their surfaces both hydrophilic and sterically stabilized using adsorbed polymers[12]. In doing so, we applied concepts familiar in the field of colloid science for the stabilization of fine dispersions against flocculation. Particles that carry adsorbed or grafted layers of polymers (Fig. 2) can display excellent stability characteristics because of the unfavourable thermodynamics for the close proximity of the adsorbed or grafted polymer chains[13]. We argued that the modification of the surfaces of particles intended for intravenous injection by a polymer to provide a hydrophilic and a steric barrier would minimize the uptake of the plasma components that caused the opsonization and recognition phenomena. In early proof-of-concept studies, we used nanoparticles constructed from the nondegradable polymer polystyrene. Block copolymers were adsorbed onto the surface of these particles to provide the necessary surface properties. We found that when these particles were injected intravenously, they were not recognized by the liver. Instead, the particles persisted within the circulation (Fig. 3). The amount of opsonic coating was also dramatically reduced. This concept of disguising particles with hydrophilic surface coatings has now been applied to biodegradable particles as well as to liposomes and emulsions[14]. The so-called 'stealth' liposome, undergoing clinical trials for tumour targeting, is a direct application of this concept[15].

The fact that particles can be engineered to avoid capture by the liver opens up opportunities for targeting within as well as outside the vascular compartment. Our own work (and that of others) has shown that particles with long circulation times can reach sites of inflammation as well as escape to the interstitium of tumours[16], as a result of the leakiness of the endothelial cells and the associated basement membrane of the local vasculature.

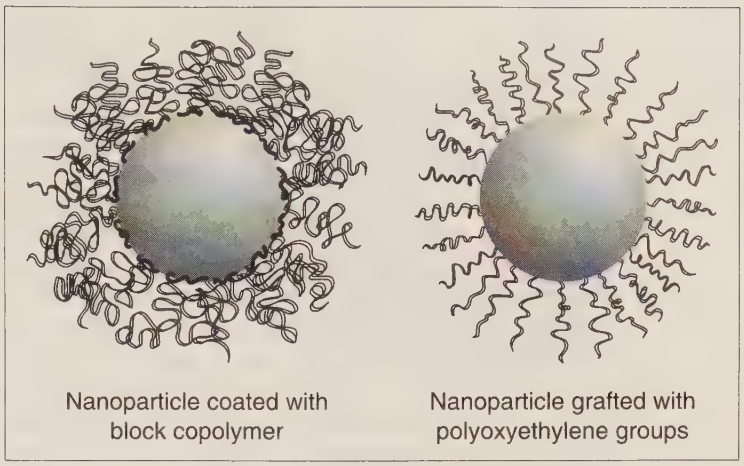

Figure 2
Nanoparticles coated with a block copolymer or grafted with polyoxyethylene groups. These surface modifications provide a steric barrier that minimizes protein conditioning and capture by macrophages.

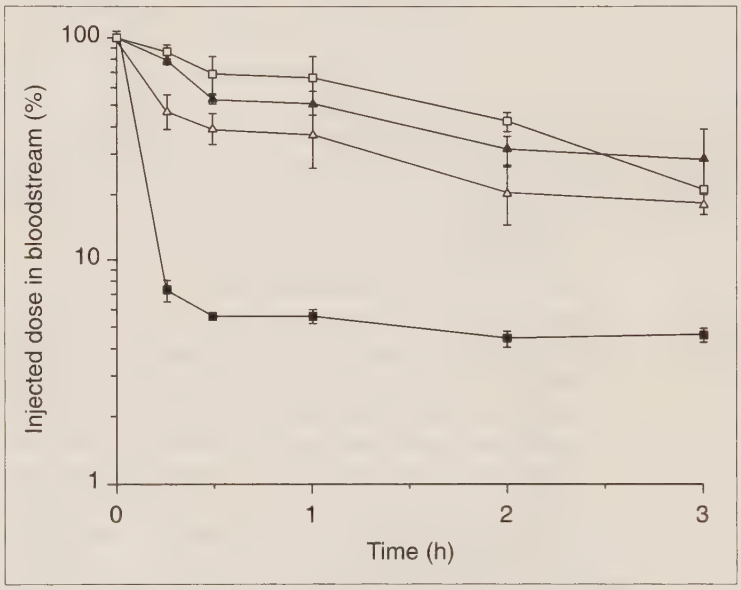

Figure 3
Blood clearance of injected nanoparticles in an animal model. The rate of removal of the particles to the liver is greatly influenced by the surface properties of the particle. Uncoated nanoparticles of the copolymer poly(lactide-*co*-glycolide) (PLGA) are cleared rapidly, while particles coated with a block copolymer or polylactide–poly(ethylene glycol) (PLA–PEG) render the nanoparticle surface hydrophilic and sterically stabilized. Data: (■), PLGA; (▲), PLGA–PEG (2:5); (△), PLGA–PLA–PEG (3:4); (□), PLGA–poloxamine 908. (Redrawn, with permission, from Ref. 14.)

Active targeting of colloidal carriers

Active targeting of particulates to achieve greater specificity (at least *in vitro*) can be achieved by the attachment of a targeting ligand to the surface of the particle conjugate. As mentioned above, monoclonal antibodies and fragments thereof, as well as sugar residues, are possible candidates. The hepatocytes of the liver can be considered to be an exemplary target. While the Kupffer cells of the liver may normally

represent an undesirable target, the parenchymal cells (hepatocytes) of the same organ can be an important target in various disease conditions, such as hepatitis, as well as in gene therapy[17]. In the latter application, it has been suggested that the liver could be used as a 'bioreactor' where administered genes could express missing factors such as growth hormones and blood factors. As a result, the targeting of plasmid DNA to the liver hepatocytes represents a major challenge in particle–drug targeting. The delivery problems to be overcome are multifaceted. It is known from studies of the anatomy of the liver that the endothelial lining of the blood vessels of the liver (sinusoids) have gaps, or fenestrations, through which nanoparticles could pass. Moreover, there is no intact basement membrane below these fenestrations. As a result there is direct access from the blood compartment to the underlying tissue (space of Disse). Thus, nanoparticles could have a close interaction with liver hepatocytes. The size of the fenestrations has been estimated to be between 100 and 150 nm, as measured by electron microscopy, but it is likely that the actual (hydrodynamic) size of the opening *in vivo* is somewhat smaller[18]. Thus, two key specifications for the targeting of a particulate carrier to the liver parenchyma via the blood circulation can be defined – a small particle size to allow access to the hepatocytes and a suitable surface character to prevent unwanted uptake by Kupffer cells.

An ability to reach the hepatocytes does not necessarily guarantee that nanoparticles will be taken up and internalized by these cells. Indeed, work in the field of liposomes would suggest that particles may need to be less than 50 nm (and preferably less than 20 nm) in diameter to be taken up by hepatocytes via non-specific endocytic mechanisms[19]. However, the liver hepatocyte is known to have receptors for a number of different ligands, including the asialoglycoprotein receptor and receptors for apoproteins on chylomicron remnants. Furthermore, extensive work has shown that it should be possible to deliver soluble polymers to hepatocytes by using conjugates that bear galactose residues[20]. Unfortunately, the simple attachment of galactose to the surface of nanoparticles does not necessarily provide the desired targeting effect, because Kupffer cells also have receptors for galactose. The specific targeting of particles to hepatocytes is believed to be possible only if galactose groups are arranged on the particle surface in a 'triantennary' fashion, in which three galactose groups have an optimal spacing that can permit the specific recognition step. A 'ligand' requirement can therefore be added to our specification for the design of a particle to be targeted to hepatocytes.

The relevance of nanotechnology to gene therapy

Gene therapy represents an exciting development in medical treatment. By the insertion of plasmid DNA into target cells, it may be possible to rectify genetic disorders, and to produce therapeutic agents in the form of peptides and proteins or antigens *in situ* to stimulate the immune system. As a result, gene therapy offers exciting opportunities and the prospect of curing diseases rather than merely treating the symptoms of a disease. Pilot Phase I experiments in human subjects have been conducted for diseases such as cystic fibrosis and muscular dystrophy, and opportunities in gene therapy are attracting interest from both small and large pharmaceutical companies[21].

Attractive targets for gene therapy are the epithelial surfaces of the lungs and gastrointestinal tract, endothelial cells lining the blood vessels, muscle myoblasts and skin fibroblasts[22]. Tumour cells, which by their very nature and their poor accessibility can represent more difficult targets for achieving an effective outcome[23], are also potential targets for gene therapy. A crucial aspect of gene therapy is the effective delivery of the (plasmid) DNA to the nucleus of a target cell (Fig. 4). This represents an important challenge in drug delivery, for not only is it necessary for the DNA to be delivered to the right cell-type but, moreover, the DNA needs to be trafficked to the correct cell compartment, the nucleus.

Three main types of gene-delivery system have been described: viral vectors, nonviral vectors (in the form of particles and polymers) and the direct injection of genetic material into tissues using so-called 'gene guns'. Viral vectors are attractive in terms of the scientific strategy of exploiting natural mechanisms. However, such systems could suffer from the inherent difficulties of effective pharmaceutical processing and scale-up, and the possibility of the reversion of an engineered virus to the wild type. There are also serious questions regarding the immunogenicity of viral vectors. Consequently, some major players in the gene-therapy field now favour nonviral vector systems even if they have a lower efficiency than their viral counterparts. Unfortunately, some nonviral vector systems are being tested in a quasiempirical fashion, both *in vitro* and *in vivo*, without proper regard to 'formulation and processing' issues, in the hope that sufficient gene expression can be achieved to give beneficial outcome.

For a nonviral vector system to be effective it is essential that the negatively charged plasmid DNA is condensed (compacted) into a nanoparticulate structure (50–200 nm in size, depending on the intended application). Advantage can also be gained if the resultant nanoparticle carries a positive charge. At present, different research groups are pursuing various strategies that include the use of cationic lipids and cationic polymers in which the interaction between the chosen cationic material and the anionic DNA results in a compacted structure[24]. Apparently, such complexes can provide both increased stability of the genetic material as well as improved uptake into target cells (Table 1). However, as yet, little detailed work has been conducted on the physical chemistry of these processes and attendant mechanisms. The final size that can be achieved for compacted plasmid DNA will depend upon the size of the original plasmid construct as well as the properties of the compacting material (and any attached surface-modifying groups or targeting ligands). The processing conditions (ionic strength,

buffers, etc.) are very important. The compaction process can be characterized by various methods and it is possible to employ many of the techniques that have been used previously for the successful evaluation of nanoparticles, such as photon-correlation spectroscopy to measure particle size, laser doppler anemometry to measure surface charge (zeta potential)[33], and probe microscopy to assess particle structure. In addition, titration microcalorimetry can be successfully used to measure the stoichiometry of the interaction between a negatively charged plasmid and a positively charged cationic material. The microcalorimetric technique can also be used to follow the effects of different polymer structures on the binding interaction as well as representing a quality-control procedure (F. Maclaughlin, S. S. Davis and M. C. Garnett, unpublished).

A design specification for DNA delivery

The nanotechnological aspects of formulations for gene therapy can be illustrated by considering the liver hepatocyte as a target. As discussed above, the compacted DNA conjugate will probably need to be less than 100 nm in size and have certain surface groupings, such as poly(ethylene glycol), that prevent unwanted recognition and others that promote interaction and internalization by hepatocytes. However, the fact that the nanoparticle conjugate will be properly recognized and internalized by the hepatocytes is not the end of the story. A particle internalized by the hepatocytes will be delivered to an endosomal compartment (and subsequently to a lysosomal compartment). In gene therapy, delivery of plasmid DNA to the endosome will be without benefit, since it is an inappropriate cellular compartment for DNA function and, moreover, presents a hostile environment to the plasmid. Therefore, it is essential that the genetic material escapes from the endosomal compartment into the cytoplasm and is presented in an episomal fashion within the nucleus so that expression may be achieved. Such escape of material from endosomes can be engineered by the incorporation of appropriate endosomolytic components. These include lytic peptides and lytic lipids[34].

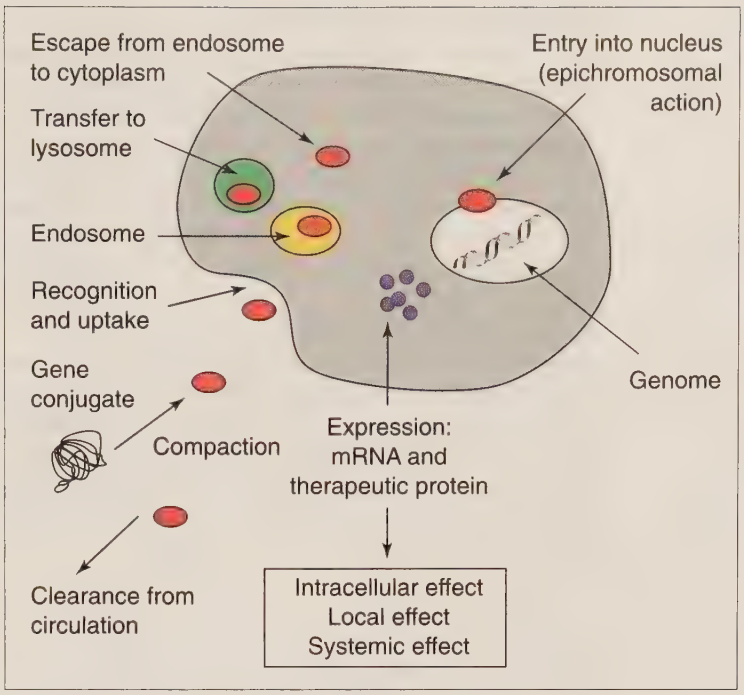

Figure 4
Schematic diagram showing the key events in the delivery of a plasmid DNA conjugate to the nucleus of a target cell and subsequent expression of a therapeutic protein. The nanoparticle needs to escape clearance by macrophages, to enter the target cell, escape from the endosome into the cytoplasm and to be presented to the nucleus. Each step is crucial for successful expression.

Each of the different factors involved in nanoparticle targeting for gene therapy (size, surface, recognition of the ligand and cell uptake of the nanoparticles, endosomal escape, episomal presentation and gene expression of the DNA) could be pivotal for a successful outcome. Consequently, gene therapy could well be the 'holy grail' in the successful clinical demonstration of the benefits of nanoparticle targeting. It is interesting and important to note that in previous studies on the use of colloidal carriers for drug targeting (such as emulsions, liposomes or microcapsules), the nanoparticle has *carried* an encapsulated drug. In the case of gene therapy the drug (plasmid DNA) is compacted to *form* the nanoparticle.

Table 1. Nonviral vectors for gene therapy based on nanoparticles and condensed DNA

System	Animal	Route	Fate	Refs
Naked DNA	Mouse	i.v.	Liver Kupffer cells	25
Cationic lipid (DOTMA)	Mouse	Aerosol	Lung deposition	26
Cationic lipid (DC-CHOL)	Man	Nasal spray		27
Polylysine conjugates (asialoglycoprotein)	Mouse	i.v.	Liver parenchyma	28
Liposomes	Rat	i.v.	Liver Kupffer cells	29
Polyvinyl derivatives	Rat	i.m.	Muscle expression	30
Polycyanoacrylate nanoparticles		Cell culture		31
Cationic polymers (dendrimers)		Cell culture		32

Abbreviations: DC-CHOL, {3-[*N*-(*N'*,*N'*-dimethylaminoethane)carbamoyl]cholesterol}; DOTMA, (1,2-dioleyloxypropyl-3-trimethyl ammonium bromide); i.v., intravenous; i.m., intramuscular.

Targeting to endothelial cells

Another example illustrating the potential for specific delivery within the vascular compartment is provided by targeting to endothelial cells. In studies in rabbits using polystyrene particles designed to minimize liver recognition and achieve long circulation times (i.e. particles of an appropriate size and bearing block copolymers), the particles were, surprisingly, found to be captured efficiently by the bone marrow[35] (Fig. 5). Extensive investigations have revealed that particle uptake was exclusively by endothelial cells. The particles were internalized through a receptor-mediated process into endosomal structures known as dense bodies. It is especially notable that this example of specific and efficient cell targeting was achieved without the attachment of targeting ligands. It may represent the first time that site-specific delivery has been achieved solely through the physicochemical process of modification of a particle surface[36].

The exact mechanism for this phenomenon of endothelial targeting has yet to be fully elucidated. It is possible that the adsorbed polymer coating on the surface of the engineered particle encourages the selective uptake of (as yet unknown) plasma component(s) to the surface of the particle during the initial particle conditioning process and that this component(s) then provides the necessary recognition event. We are clearly keen to identify this putative recognition factor. If such endothelial targeting can be shown to be a general phenomenon, it might then be possible to achieve targeting of particle systems (and particularly plasmid DNA) to a wide variety of endothelia. Kreuter *et al.*[37] have recently suggested that nanoparticles coated with the nonionic polymer polysorbate 80 were able to interact strongly with the blood–brain endothelial cells of mice. The authors were unclear as to whether the stimulus for this effect was the polysorbate coating alone or possibly other blood 'adsorbants' induced by the polysorbate coat. It is now appreciated that endothelia are relatively heterogeneous, and it is likely that different surface markers can be identified for various organ sites. This could permit targeting to specific organs by using appropriate particle conjugates based upon passive targeting processes. Such an approach contrasts with the use of the more complex process of active targeting via attached ligands and monoclonal antibodies, such as the successful targeting of DNA conjugates to the endothelia of the lung using monoclonal antibodies[38].

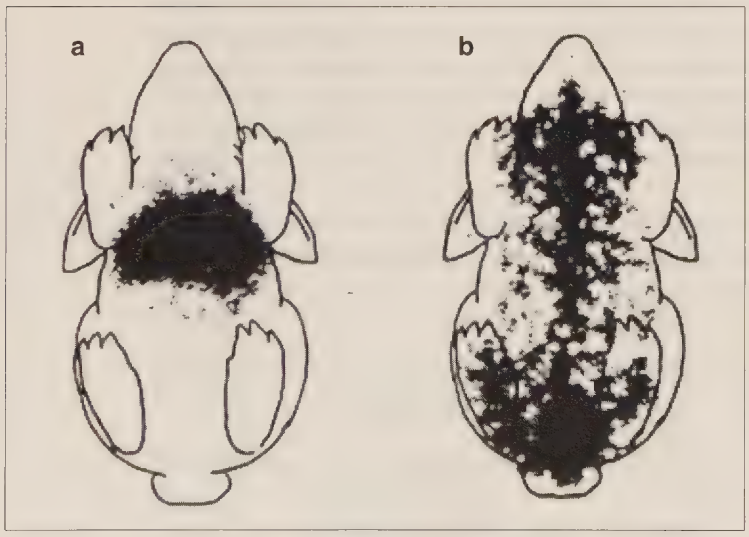

Figure 5

The uptake of nanoparticles in the bone marrow of the rabbit as measured by gamma scintigraphy. Coated and uncoated particles of 60 nm diameter were labelled with ^{131}I. Scintiphotos were taken 24 h after dosing. Coated particles are taken up by the liver (**a**), whereas uncoated particles are taken up by the bone marrow (**b**).

Delivery to the spleen

The spleen can be involved in certain disease conditions, and also has a well-known role in the immune response. It can be targeted readily using nanoparticles that have been engineered to avoid liver capture and display extended circulation times. For both nanoparticles and liposomes it has been found that larger particles (i.e. those greater than about 200 nm in size) that display long circulation (because they bear a hydrophilic and sterically stabilizing layer) do not stay in the circulation indefinitely. Instead they are filtered from the blood circulation by the spleen[39]. The extent of uptake is related to particle size; the larger the size the greater the capture. Smaller particles (less than 100 nm) do not accumulate in the spleen, apparently because they are too small to be captured by the filtration mechanism. Interestingly, we have found that, while the process of particle capture by the spleen is one of filtration, 24 h after splenic capture, non-degradable particles are subsequently found in splenic macrophages. Whether this transfer process can be exploited for improved antigen presentation has yet to be investigated[40].

Targeting to regional lymph nodes

The lymphatic compartment represents a secondary circulation system that might be exploited for drug targeting, diagnostic imaging and perhaps for gene therapy using nanoparticulate systems. The site of uptake is the lymph nodes, which act as filters within the lymphatic system. The lymph nodes can have a vital role in the staging of cancer, HIV infections and immune response.

Anatomical considerations suggest that it could be extremely difficult to reach the lymphatics and the regional lymph nodes following intravenous administration. However, there is some evidence to indicate that very small particles (i.e. those 5 nm or less in size) do escape from the vascular system to the lymphatics through high endothelial venules[41]. This escape process may be enhanced by the use of polymers such as dextran, because in certain species (e.g. the rat) dextran is known to cause a sensitization reaction, leading to greater capillary leakiness. There is also the possibility that larger particles can be transported by cells to the regional lymphatics, for example after sequestration by lymphocytes, rather than by the direct extravasation of individual particles.

A more efficient way of reaching local draining lymph nodes is via the process of interstitial (e.g. subcutaneous) administration. As before, nondegradable polystyrene nanoparticles have been used to provide a suitable material for proof-of-concept investigations[42]. Particles of different sizes and surface character have been examined in the rat model and it has been demonstrated that particles less than 100 nm in size are preferable for such targeting. Generally, the phrase 'the smaller the better' applies for particle movement from the injection site through the lymphatics to the regional lymph nodes. It has also been discovered that if particles are hydrophobic in nature then they are retained for long periods at the injection site and do not accumulate to a significant extent in regional lymph nodes. Conversely, if the particles are very hydrophilic then they are able to leave the injection site and travel into the lymphatics. However, they are not captured by the lymph nodes and pass through to the general circulation. Only particles that are intermediate in their surface properties appear to have the correct characteristics to provide good lymph-node accumulation (Fig. 6). The best targeting to regional lymph nodes achieved so far, using model systems, has been greater than 30% regional accumulation, based on administered dose. The particles are found in the resident lymph-node macrophages. We are now exploiting this phenomenon for the development of diagnostic contrast agents in magnetic resonance imaging, and in computed tomography. The targeting of compacted DNA conjugates to the lymph nodes draining neoplastic sites could be relevant in cancer therapy.

Drug loading

The loading of nanoparticulate systems with a suitable amount of drug is in itself an area of special importance. The drug, or specially synthesized derivative, needs to have a high affinity for the carrier to give good loading and to be retained with the carrier until it reaches the target tissue. The entrapment of drugs in solid polymeric matrices or the use of lipid-soluble esters in liposomes or emulsions represent well-tried strategies[43]. Ion-exchange and pH-dependent loading methods have also been employed[44].

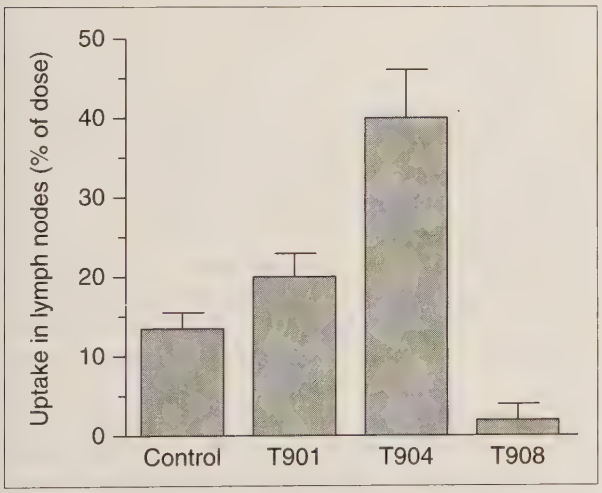

Figure 6

The effect of surface character on the uptake of 60 nm nanoparticles in the regional lymph nodes of the rat following interstitial (subcutaneous) administration. Three different block copolymers of the polyoxamine series (polyoxyethylene/polyoxypropylene) were used as coating agents. The surface hydrophobicity (as measured by hydrophobic-interaction chromatography) varies in the order 901>904>908. (Redrawn, with permission, from Ref. 33.)

Conclusions

Nanoparticles can be used to target different organ sites in the body. In each case the design of a clinically effective particle system will be dependent on a proper understanding of the relevant aspects of colloid science, biophysics and molecular biology. Nanoparticle conjugates in the form of compacted plasmid DNA, with appropriate particle size and surface properties, could be of great importance in the emerging field of gene therapy.

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Peptide nucleic acids: expanding the scope of nucleic acid recognition

David R. Corey

Peptide nucleic acids (PNAs) are DNA analogs containing neutral amide backbone linkages. PNAs are stable to degradation by enzymes and hybridize to complementary sequences with higher affinity than analogous DNA oligomers. PNA synthesis employs protocols derived from solid-phase peptide synthesis, making the methodology straightforward and flexible. PNAs are being incorporated into an expanding set of applications, including genome mapping, the identification of mutations and measurement of telomere length. The growth in the popularity of PNAs as a tool for nucleic acid recognition should accelerate as the properties of PNAs become more familiar.

The recognition of complementary DNA or RNA sequences by oligonucleotides is a central feature of biotechnology. Such recognition underlies widely used experimental techniques and diagnostic protocols, and makes it possible to consider antisense- or antigene-based inhibition as an approach to therapeutics. Given the pervasive use of oligonucleotides, improvements in the rate, affinity or specificity of oligonucleotide recognition are fundamentally important. Many laboratories have appreciated this fact and developed novel oligonucleotide chemistries to enhance hybridization, increase resistance to nuclease digestion and improve membrane permeability[1]. Most of the novel motifs contain relatively modest changes to either the nucleotide bases or to the phosphate backbone. However, one motif, peptide nucleic acids (PNAs)[2,3], represents a dramatic departure from standard oligonucleotide chemistry, and it is becoming clear that imagination will be the primary limitation to the development of new applications for PNAs.

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PNAs have been the subject of recent reviews[4,5], and I will focus here on practical considerations for their synthesis, on their physical properties and on how these properties offer significant advantages for developing new applications for nucleic acid recognition.

PNA synthesis

PNAs are DNA analogs in which 2-aminoethylglycine linkages replace the normal phosphodiester backbone[2–5]. A methylene carbonyl linker connects standard nucleotide bases to this backbone at the amino nitrogens (Fig. 1). This chemistry has three important consequences: firstly, in contrast to DNA or phosphorothioate oligonucleotides, PNAs are neutral molecules (I will address the influence of the absence of charge in subsequent sections); secondly, PNAs are achiral, which avoids the need to develop a

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PII: S0167-7799(97)01037-8

stereoselective synthesis; and thirdly, PNA synthesis uses standard Boc (Ref. 6) or Fmoc (Ref. 7) protocols for solid-phase peptide synthesis.

Laboratories can obtain PNA monomers or ready-made oligomers from PerSeptive Biosystems (Framingham, MA, USA) or from suppliers holding a license from PerSeptive Biosystems. PNA syntheses by either Boc or Fmoc protocols are straightforward using manual or automated protocols. Our laboratory finds manual synthesis to be simple, inexpensive and efficient[8]. It lends itself readily to the production of chemically modified PNAs or the simultaneous synthesis of families of closely related PNAs. Standard precautions, such as the use of fresh, dry reagents during the coupling steps, generally lead to successful syntheses. Manual synthesis will be especially advantageous for laboratories that have small budgets for PNA synthesis or that wish to synthesize a pilot series of PNAs to test the feasibility of a project*. Laboratories with the experience or the resources to support automated synthesis may prefer that approach, especially if they foresee the need for synthesis of large numbers of PNAs.

As with peptide synthesis, the success of a particular PNA synthesis will depend on the properties of the chosen sequence. For example, while in theory PNAs can incorporate any combination of nucleotide bases, we find that the presence of adjacent purines can lead to deletions of one or more residues in the product. In anticipation of this difficulty, we usually repeat the coupling of residues likely to be added inefficiently. We purify PNAs by reverse-phase high-pressure liquid chromatography[8] and achieve yields and purity of product similar to those observed during the synthesis of peptides. PNAs exhibit excellent stability to the deprotection conditions required for either Boc or Fmoc synthesis.

It may be desirable to modify PNAs for a given application (Table 1). Modifications can be accomplished by coupling amino acids during solid-phase synthesis or by attaching compounds that contain a carboxylic acid group to the exposed N-terminal amine. Alternatively, PNAs can be modified after synthesis by coupling to an introduced lysine or cysteine. The ease with which PNAs can be modified facilitates optimization for better solubility or for specific functional requirements. Once synthesized, the identity of PNAs and their derivatives can be confirmed by mass spectrometry.

Regardless of how PNAs are obtained, investigators should anticipate that several will be needed to achieve experimental aims. PNA properties, such as solubility and target recognition, will not always be predictable, especially for laboratories lacking experience with PNAs. We have generally needed to redesign our initial PNAs at least once before obtaining oligomers with the desired properties. Laboratories unwilling or unable to make this commitment of resources should critically re-examine their plans for PNAs, balancing the potential complications with the potential for useful insights.

*Detailed protocols for the manual synthesis of PNAs are available from the author upon request.

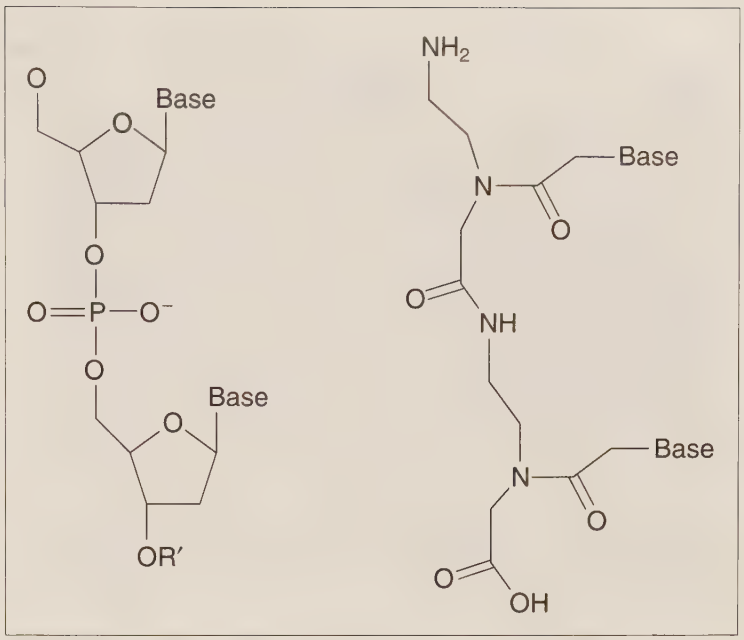

Figure 1
Chemical structure of DNA (phosphate backbone; left) and PNA (amide backbone; right) oligomers.

Physical properties of PNAs

In contrast to DNA and RNA, which contain negatively charged linkages, the PNA backbone is neutral. In spite of this dramatic alteration, PNAs recognize complementary DNA and RNA by Watson–Crick pairing[22], validating the initial modeling by Nielsen *et al.*[2] PNAs lack 3′ to 5′ polarity and can bind in either parallel or antiparallel fashion, with the antiparallel mode being preferred[22]. Nuclear magnetic resonance studies demonstrate that PNAs form a complex with RNA that is similar to an A-type helix[23], whereas a PNA–DNA duplex has elements of both A- and B-type structure[24]. Crystallography has revealed that the 2:1 PNA–DNA complex forms a unique multistranded triplex structure and confirms that both Hoogstein and Watson–Crick base-pairing are present[25], and that a PNA–PNA complex possesses a unique structure[26].

Table 1. Modifications to PNAs

Type of modification	Aim/use	Refs
Acridine	Oligomer stabilization	2
Protein	Specific DNA cleavage	8
Modified backbone chemistries	–	9
DNA	–	10–12
Peptide	Protein-kinase-C substrate	13
Peptide	His6-based purification	14
Peptide	Metal-dependent DNA cleavage	15
bis-PNA	Enhanced hybridization	16
Biotin	Affinity purification	17–19
Fluorescein	Visualize hybridization	20,21

Hybridization of DNA oligonucleotides to DNA and RNA is destabilized by electrostatic repulsion between the negatively charged phosphate backbones of the complementary strands. By contrast, the absence of charge repulsion in PNA–DNA or PNA–RNA duplexes increases the melting temperature (T_m) and reduces the dependence of T_m on the concentration of mono- or divalent cations[2]. The lack of repulsion enhances the rate of PNA hybridization to sequences containing inverted repeats within double-stranded supercoiled DNA, increasing the association constant 50 000 times ($k_a^{PNA} \approx 5 \times 10^5\ M^{-1}\ s^{-1}$) relative to that for analogous DNA oligomers[27]. The enhanced rate and affinity of hybridization are significant because they are responsible for the surprising ability of PNAs to perform strand invasion of complementary sequences within relaxed double-stranded DNA, an intriguing phenomenon that is discussed below. In addition, the efficient hybridization at inverted repeats suggests that PNAs can recognize secondary structure effectively within double-stranded DNA. Hybridization of PNAs to single-stranded DNA is also rapid ($k_a \approx 10^6\ M^{-1}\ s^{-1}$; Ref. 22), although in this case the rate of association of PNAs is equivalent to that observed for hybridization of DNA oligonucleotides to complementary sequences, due to the fact that the negative charge of only one target strand is present to hinder hybridization by DNA oligomers. Enhanced recognition also occurs with PNAs immobilized on surfaces, and Wang *et al.* have shown that support-bound PNAs can be used to detect hybridization events[28].

One might expect that tight binding of PNAs to complementary sequences would also increase binding to similar (but not identical) sequences, reducing the sequence specificity of PNA recognition. As with DNA hybridization, however, selective recognition can be achieved by balancing oligomer length and incubation temperature. Moreover, selective hybridization of PNAs is encouraged by PNA–DNA hybridization being less tolerant of base mismatches than DNA–DNA hybridization. For example, a single mismatch within a 16 bp PNA–DNA duplex can reduce the T_m by up to 15°C (Ref. 22). This high level of discrimination has allowed the development of several PNA-based strategies for the analysis of point mutations[28–32].

High-affinity binding provides clear advantages for molecular recognition and the development of new applications for PNAs. For example, 11–13-nucleotide PNAs inhibit the activity of telomerase, a ribonucleoprotein that extends telomere ends using an essential RNA template, while the analogous DNA oligomers do not[33] (Fig. 2). Presumably, binding by the latter to the telomerase template is too weak to block polymerization. Inhibition is highly dependent on the complementarity of the PNAs to the telomerase template, and PNA scanning, in which relatively short PNAs probe overlapping nucleotide sequences, has identified nucleotides within the telomerase template that are determinants for optimal inhibition (S. E. Hamilton and D. R. Corey, unpublished).

While the neutral backbone linkages strengthen the binding of PNAs to complementary nucleic acid sequences, the lack of a phosphate backbone removes the electrostatic interactions essential for protein binding. This fact has several implications for the use of PNAs. The most obvious is that PNAs are not susceptible to digestion by proteases or nucleases because enzymes cannot recognize their hybrid chemistry, affording PNAs a long half-life in serum[34]. More subtly, PNAs appear to have little ability to interact either sequence-specifically or otherwise with proteins that possess an affinity for DNA or RNA. For example, while short DNA duplexes analogous to the promoters for SP6 or T7 RNA polymerase are able to bind polymerase and inhibit transcription with high selectivity, and phosphorothioate duplexes are potent but non-sequence-selective inhibitors, PNA duplexes neither bind measurably to polymerase nor inhibit transcription[35]. Unwanted interactions between proteins and nucleic acids are a major complication to the use of phosphorothioate oligomers in antisense research, and the lack of such interactions may improve the ability of PNAs to locate target sequences selectively within cells. Neutral PNAs are more hydrophobic than analogous DNA oligomers, and this can lead to difficulty solubilizing them at neutral pH, especially if the PNAs have a high purine content or if they have the potential to form secondary structures. Their solubility can be enhanced by attaching one or more positive charges to the PNA termini[2].

To date, there have been no reports of the successful crossing of cell membranes by PNAs. Studies on phospholipid vesicles and whole cells have shown that PNAs do not readily cross membranes, suggesting that passive diffusion is not a viable mechanism for the introduction of PNAs into the nuclei of living cells[36,37]. However, the adaptability of PNA synthesis to the design of modified PNAs will allow the pursuit of multiple strategies for the introduction of PNAs and their derivatives into cells. While the current absence of techniques for intracellular delivery does not detract from the potential of PNAs for *in vitro* applications, the development of methods for their efficient uptake will be an outstanding achievement for the field.

PNA hybridization by strand invasion

One of the most interesting and instructive properties of PNAs is their ability to recognize sequences within duplex DNA by strand invasion[2,3] (Fig. 3). Strand invasion is doubly remarkable because PNAs must initiate recognition at target sequences that are already base-paired and then continue binding, overcoming entropic considerations favoring reformation of the parent duplex. Early studies recognized strand invasion by PNAs through gel retardation of hybridized complexes and through nuclease or chemical nicking of the displaced strand[2]. Complex formation has since been confirmed by electron microscopy[38], affinity cleavage[15] and altered circular dichroism[39], and appears to be under kinetic control[40].

Initial work targeted PNAs to polypurine–polypyrimidine tracts. Surprisingly, PNAs did not hybridize by standard triplex formation, but formed four-stranded complexes consisting of a target DNA strand, a Watson–Crick-paired PNA strand, a Hoogstein-paired PNA strand, and a displaced DNA strand[4]. Upon recognition of this, Griffith *et al.* demonstrated enhanced recognition by bis-PNAs that contained two strands connected by a lysine-containing linking sequence[16]. Presumably the initiation of strand invasion by PNAs at polypurine–polypyrimidine sequences is facilitated by rapid hybridization of PNAs to transiently unpaired duplex targets. If this hybridization persists long enough (a variable that is dependent on exact complementarity of the PNA to the DNA[40]), a second PNA can bind by Hoogstein pairing and stabilize the four-stranded complex. PNAs bind by strand invasion to sequences containing inverted repeats[8,27] and triplet repeats[19], suggesting that sequences possessing a tendency to form non-B-type structures are particularly suitable targets for recognition, and that strand invasion is not restricted to homopurine–homopyrimidine sequences. Recent studies have also shown that PNAs of mixed sequence can invade sequences lacking secondary structure, albeit at a slower rate[41].

The addition of millimolar concentrations of divalent cations prevents strand invasion by PNAs *in vitro*, presumably because increased ionic strength stabilizes the parent duplex, and this has been thought to be a limitation on PNA use. However, it is important to emphasize that, *in vitro*, the cation concentration can be controlled during hybridization. *In vivo*, the situation is much more complex, and supercoiling, transcription, DNA secondary structure and ionic strength will combine to dictate whether or not PNAs can bind by strand invasion. This belief is supported by comprehensive *in vitro* studies that reveal that supercoiling and ionic strength are critical determinants of the efficiency of strand invasion[42]. As will be discussed below, Allfrey and colleagues have reported that PNAs can recognize sequences within permeabilized cells,

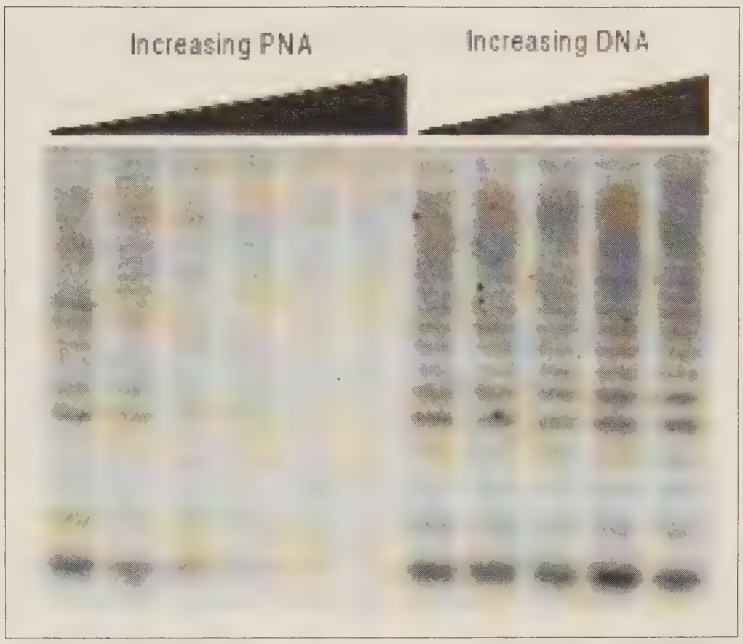

Figure 2

Effect on strand elongation by human telomerase of the addition of analogous PNA (0.0003, 0.003, 0.03, 0.3, 3.0 and 30.0 μM) and DNA (0.003, 0.03, 0.3, 3.0 and 33 μM) oligonucleotides as measured by the PCR-based TRAP assay[33]. Strand elongation is inhibited by PNA, but not by the analogous DNA oligonucleotides. PNA and DNA oligomers were complementary to the RNA template of telomerase. Image supplied by Anne Pitts (University of Texas Southwestern Medical Center, Dallas, USA).

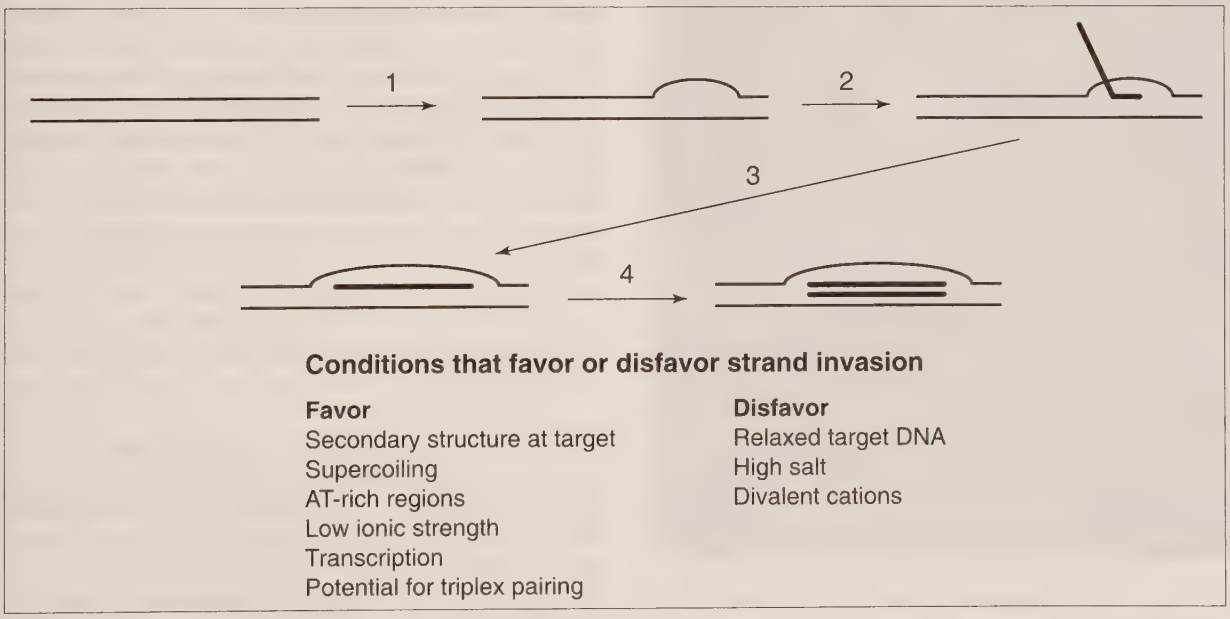

Figure 3

Possible intermediates during strand invasion of duplex DNA. (**1**) Duplex target transiently becomes single-stranded. (**2**) PNA takes advantage of its ability to hybridize rapidly and stably to initiate binding. (**3**) PNA oligomer completes strand invasion; high-affinity PNA hybridization discourages reformation of the parent duplex. (**4**) Pairing at homopurine–homopyrimidine sequences can be further stabilized by triplet formation to form a 2:1 PNA–DNA complex. Conditions that favor or disfavor strand invasion are shown.

Table 2. Development of applications for PNAs

Application	Year	Refs.
DNA strand invasion	1991	2
Antisense inhibition	1992	3
S1-Targeted DNA cleavage	1993	44
Inhibition of restriction-enzyme cleavage	1993	45
Mutation analysis	1994	46
Promoters of transcription	1994	47
Delivery through blood–brain barrier	1995	18
Nucleic acid purification	1995	14
DNA cleavage by attached nuclease	1995	8
Isolation of transcriptionally active genes	1995	19
Enhanced PCR amplification	1995	48
Blocking of transcription factor binding	1995	49
Monitoring telomere length	1996	20
Inhibition of human telomerase	1996	33
Complementary assisted hybridization	1996	27
Detection of mutations	1996	29,30
Genome cleavage	1996	50
Biosensors	1996	28
Arrest of transcription at CAG repeats	1996	43
Metal-dependent cleavage of DNA	1996	15
Transcription-mediated binding	1996	51
Translation inhibition by PNA clamps	1996	52
In situ hybridization	1996	53
High-throughput screening of RNA	1996	31
Alternative to Southern blotting	1996	32

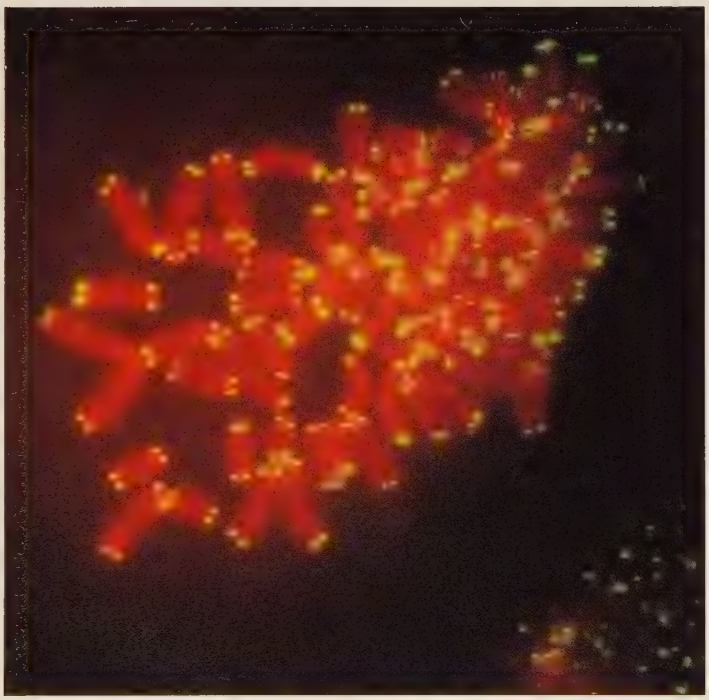

Figure 4
Telomere recognition by PNAs – *in situ* hybridization of fluorescein-labeled PNA to telomeres. This image was produced by Steven Sherwood (Geron Corporation) using protocols adapted from Lansdorp *et al.*[20] Low photobleaching and an excellent signal-to-noise ratio make it possible to quantitate telomeric repeats on individual chromosomes.

suggesting that strand invasion by PNAs can occur under conditions that are near-physiological[43].

Applications for PNAs

Standard DNA oligonucleotides already function effectively in many methods, and so the question arises of whether the advantages of PNAs justify the development of new protocols. To be more than just a laboratory curiosity, PNAs must either offer the potential for significant improvement of existing protocols or permit the development of new procedures that would not otherwise have been possible. It is reasonable to expect that PNAs might meet these requirements. The greater association constants for PNA hybridization will increase the rate of recognition and decrease the concentration of oligomer necessary, thus improving throughput and reproducibility while reducing cost. The enhanced affinity of PNA binding will allow recognition of binding sites at lower ionic strengths and higher temperatures than before. An increasing number of applications have been described (Table 2), confirming the potential for PNAs to facilitate the development of novel methodologies. Two specific applications are offered below as case studies for the use of PNAs.

Determination of telomere size

Standard protocols for telomere length determination employ Southern blot analysis of genomic DNA and provide a range for the telomere length of all chromosomes present. Methods have been developed to monitor the *in situ* hybridization of fluorescein-labeled DNA oligonucleotides to telomeric repeats, but these procedures provide only qualitative information. To monitor telomere length quantitatively, Lansdorp *et al.* adopted the use of fluorescein-labeled PNAs[20]. This methodology yields clear results (Fig. 4), and the data obtained allow accurate estimates of telomere length to be made[20]. It is likely that variations of this approach can be applied to other repetitive sequences to achieve similar results. In addition, PNA recognition has been exploited to identify efficient alternatives to Southern blot analysis for discriminating between hybridization to mutant and nonmutant target sequences[29,32]. A similar report has described the use of PNAs for *in situ* hybridization to mRNA, and has shown that, relative to hybridization of DNA oligonucleotides, PNA hybridization is faster and requires a lower concentration of probe[53].

Strand invasion of transcriptionally active genes

Lansdorp *et al.* solved the problem of accessing sequences within chromosomal DNA by denaturing the DNA. Findings by Allfrey and colleagues, however, suggest that strand invasion will occur spontaneously at sequences within chromosomal DNA[19,43]. These studies targeted PNAs to triplet repeats of the nucleotides CAG and used this recognition to purify transcriptionally active DNA[19] and to inhibit transcription[43]. These results indicate that efficient strand invasion is not restricted to polypurine–polypyrimidine

sequences or inverted repeats but can occur at other sequences as well. CAG repeats have some tendency towards partially single-stranded structures, and secondary structure or active transcription may have facilitated the initiation of base-pairing at the target sequence. Interestingly, inhibition of transcription is also observed within permeabilized cells[43], indicating that strand invasion can occur under physiological conditions of ionic strength. This result suggests that if PNAs can be delivered within cells then they will have the potential to be general sequence-specific regulators of gene expression.

Concluding remarks

It is tempting to conclude that 'anything DNA can do, PNAs can do better'. This is a simplification, of course, since PNAs cannot act as primers nor as templates for polymerases and they do not appear to have much potential to interact with proteins that recognize nucleic acids. However, PNAs do possess undeniable advantages for the recognition of complementary DNA and RNA sequences, and are a major advance in molecular recognition rather than being a mere curiosity. Exploiting these advantages will not always be simple. PNAs are a novel chemical motif, and investigators wishing to initiate PNA-dependent projects should consider their resources, the properties of PNAs and the applicability of conventional oligonucleotides. If the use of PNAs is not considered carefully, it is likely that the investigator will achieve little beyond complaints regarding poor solubility and high cost. However, for those willing to invest a sustained effort, PNAs provide the basis for significant technological improvements and novel scientific insights.

Acknowledgements

I thank Mieczyslaw Piatyszek (Geron Corporation, Menlo Park, CA, USA), Donald Doyle and Susan Hamilton for thoughtful comments on this manuscript and Steven Sherwood and Geron Corporation for supplying Fig. 4. This work was supported by a grant from the Robert A. Welch Foundation (I-1244) and an award from the CaP CURE association. The author is an Assistant Investigator with the Howard Hughes Medical Institute.

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Expanded-bed adsorption in industrial bioprocessing: recent developments

Rolf Hjorth

Expanded-bed adsorption allows the capture of proteins from particle-containing feedstocks without prior removal of particulates, thus enabling clarification of a cell suspension or cell homogenate and the concentration of the desired product in a single operation. This usually results in higher product recovery in a shorter time period. Process development and scale-up of an expanded-bed operation is convenient because both the adsorbent and the equipment are similar to those in conventional packed-bed chromatography. This article reviews the recent developments in expanded-bed adsorption technology and the range of applications that are now being achieved.

The recovery of proteins from various recombinant host organisms is a crucial procedure in the production of proteins for therapeutic and diagnostic use. The initial steps in the production process usually involve several unit operations to remove particulate matter and to concentrate the desired product. It is important that these initial recovery steps are carried out rapidly to minimize the risk of proteolytic breakdown of the product. Furthermore, the number of steps should be as few as possible to avoid product losses. The traditional techniques employed for performing these initial steps have been centrifugation and/or microfiltration[1]. These have been in use for many years and are regarded as standard in the biotechnology industry. Nevertheless, they suffer from some drawbacks. For example, the degree of clarification in an industrial centrifuge is usually in the range of 10^2–10^3 in terms of cell clearance[2,3]. For this reason, centrifugation is usually combined with a microfiltration step to obtain a particle-free solution that can be further purified by traditional packed-bed chromatography[3]. Although microfiltration yields cell-free solutions, in many cases the flux of liquid per unit membrane area is dramatically decreased during the filtration process[4]. Combined centrifugation and filtration operations often result in long processing times or the use of comparatively large units.

To overcome these problems, fluidized bed processes were introduced for the recovery of antibiotics[5] and other low molecular weight compounds from cell-containing fermentation broths, even at very large scales[6]. Despite the successful application of fluidized beds for the recovery of low molecular weight compounds, their use for the recovery of proteins has been reported only in a very few cases. The main reason for this has been the lack of suitable adsorbents and equipment for such processes.

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Expanded-bed adsorption

In the expanded bed, a particulate adsorbent in a column is allowed to rise from its settled state by applying an upward flow. This increases the space between the adsorbent particles, allowing cells and cell debris to pass through without blocking the bed (Fig. 1). An important feature of the expanded bed compared with a conventional fluidized bed is its low backmixing, achieved by the proper design of the adsorbent and the column. The particle size distribution of the adsorbents gives a segregated or stratified bed in expanded mode. This means that the larger adsorbent particles are found in the lower part of the expanded bed and the smaller adsorbent particles in the upper part (Fig. 1). Usually, adsorbents for expanded beds have particle sizes ranging from 50 to 400 μm. Smaller particles result in overexpansion at low flow velocities and larger particles require very high flow velocities to expand the bed sufficiently. In the first case, the overall productivity of the process will be low, and in the second case, protein binding is impaired due to restricted diffusion into the adsorbent particles. Densities in the 1.1–1.3 g ml^{-1} range are required to be able to operate the expanded bed at flow velocities that result in high productivity.

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PII: S0167-7799(97)01045-7

To obtain an expanded bed with low backmixing, a plug-flow profile through the column is necessary. This is achieved by generating a pressure drop over the liquid inlet of the expanded-bed column. A review of the early developments of expanded beds and their function has recently been published[7].

Several attempts have been made to improve the process by designing systems that minimized the backmixing in the expanded-bed column, thereby increasing the efficiency of the adsorption process. These included the development of segmented columns[8] or stabilization of the expanded bed using magnetic fields[9]. Other developments included the use of adsorbents for packed-bed chromatography in combination with a column design that generated plug flow. These latter improvements have shown that it is feasible to reduce the backmixing to a degree where expanded beds can be efficiently used[10,11]. However, none of these approaches was able to meet the requirements of the biopharmaceutical industry in terms of productivity and hygiene.

Adsorbents and equipment for expanded-bed adsorption

Over the last ten years, several different materials have been applied for the recovery of proteins in expanded-bed processes. Such materials were usually made of composites like dextran–silica[12] or cellulose–titanium-dioxide[13]. More recently, a hydrophilized perfluoropolymer adsorbent was developed for use in expanded beds[14]. All of these materials are of limited use either because of their low protein-binding capacity or because of their inability to withstand the high pH necessary for cleaning. The development of purpose-designed adsorbents based on an agarose–quartz composite resulted in a material with a high protein-binding capacity that was also stable at a high pH[15,16]. More recently, an adsorbent based on an agarose–metal core composite, to which protein A was attached, was developed for the recovery of IgG. A summary of the properties of adsorbents specially designed for use in expanded beds is given in Table 1.

The equipment used in an expanded-bed process is similar to that used in packed-bed chromatography processes. The only exception is the design of the expanded-bed column, which has two features that distinguish it from a conventional chromatography column – the liquid inlet and the top adapter. As there is no pressure drop over the expanded bed, one has to be generated through a perforated plate in the liquid-distribution inlet on the column. Furthermore, in an expanded-bed column, the top adapter is moveable to allow the column to be operated at different bed heights, which is necessary during the different stages of operation of the expanded-bed process.

Development of an expanded-bed process

Four different experimental stages are generally required to study the parameters that are important for the successful development of an expanded-bed process from laboratory to production-scale. These stages are:

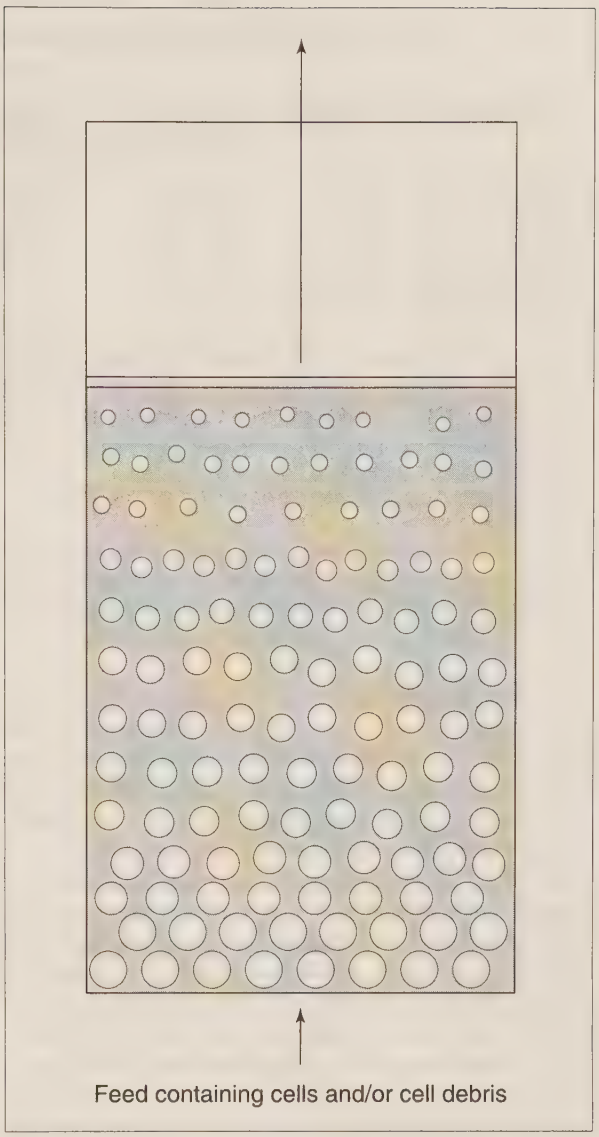

Figure 1
The principle of an expanded bed. The increased space between the adsorbent particles allows cells and cell debris to pass unhindered through the bed. The arrows indicate the flow direction in expanded-bed mode.

(1) small-scale experiments in packed-bed mode;
(2) small-scale experiments in expanded-bed mode;
(3) pilot-scale experiments in expanded-bed mode;
(4) production-scale experiments in expanded-bed mode.

The first step studies the influence of product characteristics on the purification process by using a small packed bed. Such experiments require the use of a clarified feedstock to avoid blockage of the chromatographic bed. At this stage the optimal conditions for binding and elution of the product are determined. In addition, it is possible to obtain an estimate of the binding capacity for the product in the presence of other proteins and impurities.

The next step in the development process is the study of an unclarified feedstock in a small-scale expanded-bed column. Here, the influence of cells and/or cell debris on the adsorption process is

Table 1. Some properties of commercially available purpose-designed adsorbents[a] for expanded-bed adsorption

Base matrix	Ligand	Particle size distribution (μm)	Mean density (g ml^{-1})	Breakthrough capacity (mg ml^{-1})	Total binding capacity (mg ml^{-1})[b]
Agarose–quartz	DEAE	100–300	1.15	35[c]	55
Agarose–quartz	SP	100–300	1.15	65[c]	75
Agarose–quartz	IDA	100–300	1.15	8[d]	40
Agarose–quartz	Heparin	100–300	1.15	ND	[e]
Agarose–metal	Protein A	80–165	1.3	20[f]	50

[a]Marketed under the trade name STREAMLINE® (Pharmacia Biotech AB, Uppsala, Sweden).
[b]Total binding capacity was determined in packed-bed mode at 50 cm h^{-1} flow velocity at 50% breakthrough, using bovine serum albumin for DEAE and IDA, lysozyme for SP and human IgG for Protein A.
[c]Determined at 300 cm h^{-1} flow velocity in expanded-bed mode (15 cm sedimented bed height) at 1% breakthrough, using the same proteins as given in [b].
[d]Determined in a packed-bed run at 300 cm h^{-1} flow velocity (5 cm sedimented bed height) at 1% breakthrough, using bovine serum albumin.
[e]Functionality was evaluated by ability to bind antithrombin from bovine plasma.
[f]Breakthrough capacity was determined using human IgG under the conditions given in [c].
Abbreviations: DEAE, diethyl aminoethyl; IDA, iminodiacetic acid; ND, not determined; SP, sulfopropyl.

evaluated. At this stage it is important to determine the amount of biomass that can be applied to the expanded bed and to assess the ability of the process to remove cells and cell debris. Data from previous studies show that it is possible to apply approximately 5% dry weight of cells when working with yeast cells[17,18] or cell homogenates from *Escherichia coli*[19]. It has also been reported that the use of expanded-bed adsorption reduces the cell concentration in the eluted fraction by a factor of 10^4–10^5 (Refs 15,16), which is at least one to two orders of magnitude higher than that achieved in an industrial centrifuge.

The third step is to verify the results obtained from the small-scale experiments at pilot scale. This usually involves three to six litres of adsorbent and 25 to several hundred litres of feedstock, depending on the nature of the feed material and the concentration of the product. At this scale, it is important to keep both the bed height and flow velocity the same as in the small-scale experiments, and to increase the cross-sectional area of the column in proportion to the feedstock volume that needs to be processed.

The final step in the development of the expanded-bed process is the establishment of the process at the production scale. Production scale is the use of a column containing 50 to 300 litres of adsorbent, enabling the processing of feedstock volumes of several thousand litres.

Operation of the expanded-bed process

The expanded-bed process is operated in a similar manner to a packed-bed process. The main difference is that the direction of liquid flow is upwards during the expansion, feedstock application and washing of the bed. These operations are usually carried out at a constant flow velocity of 300 cm h^{-1}. A lower flow velocity might be used if the viscosity of the feedstock is very high, to prevent the bed from expanding too much. Another regime for feedstock application has been described by Chang and Chase[20], in which they applied the feed at a flow velocity that resulted in a doubling of the bed volume. Under specific conditions, they achieved a higher productivity from the expanded-bed process.

After unbound material has been washed out, the flow is stopped and the bed allowed to settle. Elution is usually performed in packed-bed mode using a downward flow at a decreased velocity, usually 100 cm h^{-1}, to keep the volume of the eluted fraction small. Elution may be carried out in expanded-bed mode, but this has been found to result in a 40% increase in the volume of the eluted fraction[16]. More detailed descriptions of the operation of expanded beds have been published[7,19].

An important stage in the operation of an expanded bed is the cleaning-in-place (CIP) procedure. CIP is performed to maintain the functionality of the adsorbent and should ensure that all impurities are removed to prevent carryover of material between batches. The CIP procedure used is dependent on the type of feedstock applied and so several have been developed[18,19,21,22]. Most CIP procedures include the use of sodium hydroxide in concentrations of 0.5 to 1 M. This treatment should be performed for a controlled period of time (3–4 h), to allow contaminants to be removed. Sodium hydroxide is usually combined with other CIP solutions, such as 10% acetic acid or organic solvents such as 20% ethanol. For ligands that do not tolerate sodium hydroxide, for example protein A, treatment with 6 M guanidinium hydrochloride is a useful alternative.

An issue related to CIP is the clearance of viruses and endotoxins from expanded beds. To our knowledge, only preliminary data has been published in this field[23]. However, it is unlikely that the results obtained with expanded beds will differ significantly from those of packed beds.

Table 2. A summary of data from selected expanded-bed adsorption processes

Feedstock	Feed volume (l)	Product	Adsorbent ligand	Adsorbent volume (l)	Purification factor	Yield (%)	Ref.
E. coli cell suspension	8	ZZ–M5	DEAE	0.3	NA	90	15
E. coli homogenate	26	Annexin V	DEAE	4.7	2	95	19
E. coli homogenate	10	FGF-Saporin	SP	0.3	20	65	27
E. coli periplasmic extract	180	Exotoxin	DEAE	4.7	2	79	28
Yeast cell suspension	2000	rHSA	SP	150	NA	87	29
Yeast cell suspension	6.4	Aprotinin	SP	0.3	3.8	76	17
Cell culture	60	Monoclonal IgG	Protein A	0.15	30	83	30
Cell culture	100	Monoclonal IgG	Protein A	4.7	NA	NA	22
Cell culture	12 000	Monoclonal IgG	SP	154	NA	95	31
Cell culture	36	Monoclonal IgG	SP	0.17	7	85	32
Cell culture	NA	rhuNGF	SP	NA	NA	95	33
Milk	5	Lysozyme	SP	0.8	8.3	89	24
Renatured inclusion bodies	16	IL-8	SP	0.3	3.9	100	26

Abbreviations: DEAE, diethyl aminoethyl; FGF, fibroblast growth factor; IL-8, interleukin 8; NA, data not available; rHSA, recombinant human serum albumin; rhuNGF, recombinant human nerve growth factor; SP, sulfopropyl; ZZ–M5, synthetic IgG binding domain–malaria M5 peptide fusion protein.

Applications of expanded-bed adsorption for protein recovery

Expanded-bed adsorption has been used in a number of processes where proteins are recovered from feedstocks containing cells and/or cell debris. Although many of these applications have been run only at laboratory-scale, several examples of processes run at pilot- and production-scale do exist. Among the processes run only at laboratory-scale, a number may need to be scaled up as they are used for the recovery of proteins with potential therapeutic applications. It is interesting to note that proteins have successfully been recovered from nontraditional feedstocks, such as milk[24,25] and solutions containing renatured protein[26], using expanded-bed adsorption. An overview of various expanded-bed processes is given in Table 2. A few examples of expanded-bed processes of industrial relevance are discussed below.

E. coli *cells and cell homogenates*

Recombinant proteins expressed in *E. coli* are usually found in the cytoplasm or in the periplasmic space. In such cases the starting material for the expanded-bed process is a cell homogenate or a cell-containing periplasmic extract. The recovery of annexin V from an *E. coli* homogenate using an ion-exchange adsorbent has been described by Barnfield Frej *et al.*[19] In this study the process was developed and scaled to pilot-scale according to the development scheme discussed above. At pilot scale, the process was capable of handling 26 l of cell homogenate in less than 2.5 h in a 20 cm diameter expanded-bed column. It is interesting to note that the feedstock application took less than 20 min. This is an important aspect, since the shorter the time the product spends in the homogenate, the lower the risk of proteolytic degradation. The yield of annexin V was approximately 95% and the purification factor was 2. This is a relatively low purification factor, but still twice the value that can be achieved by centrifugation or filtration. Significantly higher purification factors (15–20) have been reported using ion-exchange adsorbents for the recovery of proteins from yeast[21] and *E. coli*[27] homogenates.

Secreted proteins expressed in *E. coli* are usually transported into the periplasm of the cells. The proteins are then released from the periplasm by osmotic-shock or a mild chemical treatment, resulting in a cell suspension containing the protein of interest. An example of an expanded-bed process for the recovery of a *Pseudomonas* exotoxin from an *E. coli* system has recently been reported[28]. In a pilot-scale experiment, the exotoxin was released from 4.5 kg of *E. coli* cells, resulting in 180 l of cell-containing feedstock, which was then applied to an expanded bed. The cycle time for the process, excluding CIP, was 2.5 h, which is about half the time needed for the clarification step in the conventional process using centrifugation.

Yeast cells

Feedstocks of yeast cell suspensions are relatively common starting materials, as the yeast cell usually exports the expressed recombinant protein efficiently into the culture medium. An example of a production-scale process using expanded-bed adsorption is the recovery of human serum albumin produced in *Picia pastoris*[29]. This process was developed using a 5 cm diameter column run in expanded-bed mode, which was then directly scaled up to a 100 cm diameter column containing 150 l of a cation-exchange adsorbent. Using this size of column it was possible to apply 1900 l of cell suspension. The yield of human serum albumin varied from 83–91% over four different runs. Use of the expanded-bed process avoided the three filtration steps required in the conventional

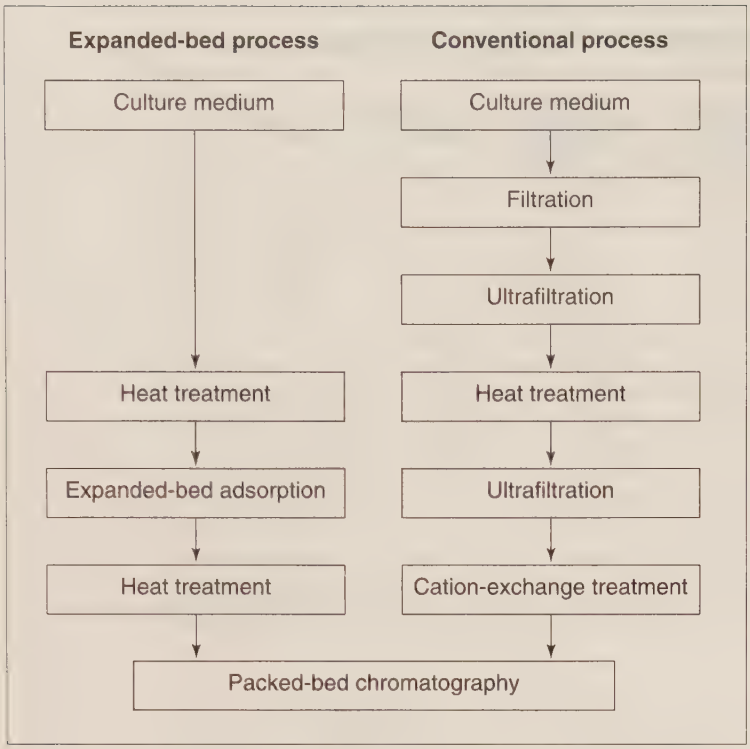

Figure 2
Process schemes for the recovery of recombinant human serum albumin produced in the yeast *Picia pastoris* using an expanded-bed process and a conventional process. (Adapted from Ref. 29.)

process (Fig. 2). It was also claimed that both product yield and purity were higher after the expanded-bed process[29].

The scaling up of expanded-bed adsorption was studied using a suspension of *Saccharomyces cerevisiae* to which bovine serum albumin was added as a model system[18]. Feedstocks of 0.75 to 440 l were applied to expanded-bed columns ranging in diameter from 2.5 to 60 cm. The performance of the process was very similar at different scales. The efficiency of a CIP procedure was investigated in the 60 cm diameter column, using both *S. cerevisiae* and *E. coli* as test organisms. After the CIP procedure, no living cells could be found on either the adsorbent or the column.

Mammalian cells

Mammalian cell culture is frequently used for the production of various therapeutic proteins, especially monoclonal antibodies. Mammalian cells are much more sensitive to changes in their environment and shear forces than *E. coli* and yeast cells. Feedstocks of mammalian cells must therefore be handled carefully to avoid cell breakage and the subsequent release of nucleic acids and proteins, including proteases.

Monoclonal antibodies are usually captured by using affinity chromatography on immobilized protein A, or by cation-exchange chromatography. Thömmes *et al.*[30] reported the use of a purpose-designed matrix to which recombinant protein A was coupled, for the recovery of a monoclonal antibody through an expanded-bed process. They were able to capture 2 g of pure mouse IgG_{2a} per cycle from an unclarified hybridoma cell culture, using a 5 cm diameter expanded-bed column. Owing to the relatively low concentration of monoclonal antibody in the cell culture, only 150 ml of adsorbent was needed to recover the antibody completely. If the full capacity of the column had been used (600 ml of adsorbent), more than 7 g of monoclonal antibody could have been recovered in a single run.

In a pilot-scale expanded-bed experiment, Jägersten *et al.*[22] recovered 40 g of an IgG_4 antibody from 100 l of myeloma cell culture in 2.5 h, using 4.7 l of immobilized protein-A adsorbent. The antibody was quantitatively recovered and its purity was comparable to that obtained using clarified feedstock and immobilized-protein-A chromatography.

A large-scale process for the recovery of a recombinant monoclonal antibody was recently presented by Zapata *et al.*[31] The initial operating conditions were determined by using 70 ml of cation-exchange adsorbent in an expanded-bed column and applying 3 l of cell-containing feedstock. From the data obtained at the laboratory scale the process was then scaled to handle 12 000 l of cell suspension. The eluted IgG fraction was concentrated fivefold and contained 95% of the applied monoclonal antibody. The cells were completely removed during the expanded-bed process. The use of a cation-exchange adsorbent for the recovery of IgG by expanded-bed adsorption has also been reported by Batt *et al.*[32]

A further example of the use of expanded beds for the recovery of products produced in mammalian cell culture is the recovery of recombinant human nerve growth factor (rhuNGF) from Chinese hamster ovary cells[33]. In this process, all the operating parameters were determined using a 2.5 cm diameter expanded-bed column containing a cation-exchange adsorbent. The yield of rhuNGF was 93–98% and the product was concentrated more than 45-fold. The process was scaled up 65-fold and the recovered rhuNGF fraction was further purified using the traditional procedure. The binding capacity of rhuNGF to the adsorbent was increased when the temperature was raised from 25 to 37°C during feed application. In addition to increasing the binding capacity, this may also have a positive effect on the cells as they are kept at optimal temperature during the initial recovery step.

Summary

Expanded-bed adsorption for the capture of proteins from feedstocks containing cells or cell debris has been used frequently during the last few years. The main reason for the increased number of examples is the availability of purpose-designed adsorbents and equipment for expanded-bed processes, making the technique efficient and compliant with the requirements of the pharmaceutical industry. Examples of processes where expanded-bed adsorption has been used to recover products from several-cubic-metre volumes of feedstocks now exist but there is still a need for the development of equipment and adsorbents that can handle even larger feedstock volumes.

There is clearly a need to develop new adsorbents with increased protein-binding capacity. The use of highly specific and stable ligands in combination with improved adsorbents will make expanded-bed adsorption even more attractive. Such improvements will allow the initial recovery of products in industrial bioprocessing to be carried out in a shorter time with a high product yield, even when very large feedstock volumes are used.

Acknowledgement

L. Hagel and J. Thömmes are acknowledged for critically reviewing the manuscript.

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Bioremediation: more than cloning and pasting DNA

Bioremediation: Principles and Applications

edited by R. L. Crawford and D. L. Crawford, Cambridge University Press, 1996. UK£65.00 hbk (xii + 400 pages) ISBN 0 521 47041 2

Metabolic engineering is one of the most important concepts in modern biotechnology. Through the judicious use of genes and promoters placed in microbial hosts, a substrate can either be taken up to produce high-added-value biomolecules or down to convert it perhaps as far as CO_2 and H_2O. Out of the two possibilities, the revolution brought about by recombinant DNA quickly took the anabolic pathway going upwards, giving rise from the early 1980s to the production and marketing of an increasingly large number of valuable chemical species, such as hormones and antibiotics.

With both pathways being technically feasible, the reasons why the anabolic pathway attracted (and still attracts) more attention in biotechnology than the catabolic (biodegradation) deserve some attention. Firstly, the scientific push towards products for the medical–pharmaceutical market connects with a pull from public demand for these products. Furthermore, there is its relative simplicity: just one gene or one sequence may lead to an extremely valuable product in a very short period of time. At the other extreme, environmental biotechnology and its key product, bioremediation, deal with slow microbial processes brought into the market almost exclusively through direct or indirect Government action. In addition, as this book clearly shows, bioremediation is by no means a technically simple thing. It requires not only the identification or construction of a proper biocatalyst for a given biodegradation problem, but also the development of large-scale processes, sometimes not unlike those used in open-cast mining, for which a broad, interdisciplinary approach is a must.

The contents of the book are organized in a sort of 'complexity gradient' that goes from the more engineering-based approach (Chapters 1 and 2) all the way down to the genetic and molecular issues addressed in the last chapter. In between, the reader finds ten excellent articles that, without

going into too much depth, provide a very complete overview of the predominant issues in bioremediation. In this respect, the book is very close to being a textbook that I would not hesitate to use as a reference in specialized courses in the university. It is interesting to note that 19 of the 23 authors are from the USA while the other four, from Israel and Finland, appear to have close ties with the USA. From this point of view, the book presents an American, practice-oriented view of the issue, which has advantages and disadvantages.

It is clear that the practice of bioremediation in the USA is far ahead of that in Europe or Japan. However, it also seems that the science behind bioremediation (i.e. biodegradation) and the efforts to construct improved microbial biocatalysts in the laboratory have been developed to a considerable extent in Europe. This is precisely one of the weaknesses of this book.

At the start, I would have liked to see at least one chapter dealing with the general issue of biodegradation of organic compounds, as a scientific problem and as a conceptual challenge, which would be a good introduction to the rest of the book. Basic issues such as the problem of upper versus lower degradation pathways, the cross-inhibition between catabolites resulting from *meta* versus *ortho* cleavage of catechol intermediates, the expression and bioavailability bottlenecks, and the jamming of catabolic pathways at their very last steps deserved a better treatment in the book. Perhaps we will have to wait for someone to write a comprehensive textbook on biodegradation that can match the excellent manuals in microbial pathogenesis published in recent years.

Although virtually all chapters of the book are worth reading, I found five of them particularly interesting. In Chapter 3, Krumholz *et al.* summarize the latest advances in biodegradation of BTEX mixtures (a combination of benzene, toluene, ethylbenzene and xylenes) under anaerobic conditions, a problem that is still difficult to tackle with the tools of molecular biology. Chapter 4, by Rosenberg and Ron, discusses the science and practice of remediation of petroleum contamination, although it almost entirely lacks any discussion of the best-characterized system for the degradation of n-alkanes, the *alk* system of *Pseudomonas oleovorants*. The rather long article by Mueller *et al.* in Chapter 5, with 350 references, addresses the degradation of one of the most recalcitrant classes of environmental pollutants, namely the polycyclic aromatic hydrocarbons or PAHs. After reading it, one is left with the feeling that there is still a long way to go before the problem can be solved. This is in contrast to the optimistic view of biodegradation of PCBs given by Unterman in the very interesting and well-written Chapter 7, one of the best. The book finishes with a very complete article by Shields and Francesconi (Chapter 11) on molecular techniques that have been applied to bioremediation. It is a pity that this chapter is placed at the end of the book, almost like an exotic piece, as it deals with some of the most exciting developments in the field, clearly shown at the Meeting on Bioremediation and Biodegradation which took place in Mallorca (Spain) in June 1996.

I would have liked to see one more chapter, on the very critical issue of the connection between the expression of catabolic genes and the physiological status of the cells. It is no secret that bacteria with an ability to degrade recalcitrant compounds frequently do not express such a phenotype under the desired conditions. The physiological regulation of catabolic pathways (e.g. catabolite control) and their energetic balance is, in fact, one of the hottest scientific issues needing to be addressed in the next few years, and could have had some consideration in the book. Emerging techniques, such as phytoremediation or biolining, could also have been discussed, at least briefly, as separate chapters or included in the Introduction.

In summary, in spite of some flaws and the fact that some of the authors' names may not be those of the specialists that one would expect, this is a generally good book that should be of interest to a wide readership, and that should be on the bookself of every scientist or engineer active in the field.

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Enzymes in your pocket

Enzymes in Molecular Biology: Essential Data

edited by C. J. McDonald, John Wiley & Sons, 1997. UK£14.99 (134 pages)
ISBN 0 471 94842 X

Today's molecular biologists have at their disposal an ever-increasing number of enzymes available from commercial sources. However, the successful design and outcome of a molecular-biology experiment or protocol is crucially dependent on the selection of the correct enzyme for an application and knowledge of its characteristics. For example, a restriction enzyme digest may be ineffective if the wrong buffer is used, the digest is performed at the wrong temperature or the enzyme will not cut methylated DNA. It can also be time-consuming to find out which enzyme is most suitable for a particular experiment, how it should be handled, what are its drawbacks and which companies supply it. The purpose of this useful book, which fits easily into the laboratory coat pocket, is to bring together all the commonly required data on enzymes and their uses. Hopefully, with this book you will

no longer need to search through the back pages of countless catalogues to find that vital piece of information.

After a general introduction dealing with the handling of enzymes, the book is arranged into three major sections – restriction enzymes, polymerases and modification enzymes, and lastly ancillary data. The restriction-enzyme section contains all the data that you could require, from a comprehensive list of all known restriction enzymes to data such as cleavage efficiencies in agarose or close to the ends of DNA molecules. The second section catalogues and describes the applications of enzymes other than restriction enzymes that modify nucleic acids. These include enzymes that are used to label, synthesize, methylate or otherwise modify nucleic acids. The third section contains information on other enzymes and proteins that are often used in molecular biology, such as the reporter enzymes chloramphenicol acetyl transferase and luciferase. This section also contains all the other miscellaneous data that one would expect to find, for example the genetic code, properties of acids and bases, and isotope half-lives. In addition there is a small section on techniques commonly used in molecular biology.

Most of the data is presented in tabular form and has been supplied by the companies that have cooperated in the production of this book. There appear to be no major omissions in the data presented but there are a few minor shortfalls. For example, perhaps the most frequently required table will be the one showing the activity of restriction enzymes in the different buffers that are supplied commercially. This information is supplied for three of the five companies suggested as sources of restriction enzymes. Why not for all five?

A slightly irritating feature of the book is its format. The text is parallel to the spine of the book, which makes it less easy to find the page you need. Also, it would have helped if the book were in ring-bound form so that it would stay open at the page you want. Despite these minor inconveniences the book will appeal both to the novice and experienced molecular biologist and should, as the authors hope, quickly become dog-eared but with any luck not fall apart!

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The PCR landscape

PCR: Essential Techniques

edited by J. F. Burke, John Wiley & Sons, 1996. UK£16.99 (153 pages)
ISBN 0 471 95697 X

This useful little book is presented in a way that makes clear its intended use – as a highly practical guide to a wide variety of techniques based on PCR. It comes as a spiral-bound book printed in 'landscape' rather than 'portrait' format, that is, with the words parallel to the spine of the book. The 31 protocols, contributed by a total of more than 60 authors, are thus intended to be read with the book open flat on the bench, with two pages of text often reading as a continuous whole. It is also no accident that the book fits snugly into the average lab-coat pocket.

The protocols are divided into eight subsections, from 'Basic techniques' through 'Gene mapping' and 'Transcriptional analysis' to 'Diagnostics and forensics'. This distribution of material is both a strength and a weakness. Organizing the protocols according to their applications makes it easy for the reader to find a protocol – and related ones – for a particular job, but makes it much harder to extract the principles underlying the recipes. For example, I found it odd that the particular difficulties of faithfully amplifying tandemly repeated DNA are not explicitly discussed but are instead tacitly represented in four different protocols. 'PCR amplification of plant DNA' for some reason appears in the section on sequencing.

The amount and detail of comment accompanying different sections is variable, with the most detailed explanations dealing with the sequencing of PCR products. Elsewhere, readers are sometimes left to provide the background for themselves. For example, in discussing human identification, there is no examination of the important practical issues in the comparative usefulness of HLA (a single locus, which may not discriminate between sibs) versus multilocus microsatellite systems. Similarly, the times given for the PCR extension step vary from 30 s to 5 min, usually with no explanatory comment.

I think it unlikely that anyone will use this collection of protocols as their only guide to PCR techniques, because the applications are in many cases tailor-made for a specific study; however, the subjects covered are so imaginatively wide-ranging that the protocols given can be used as useful starting points for home-made adaptations, particularly when venturing into unfamiliar types of experiment. Of the important applications of PCR, it was good to see sections dealing with LCR, single-cell cDNA synthesis and RDA, and only long PCR and PCR at the single-molecule level are unrepresented in this book. The virtue of the book is in the collected wealth of detailed practical advice distilled from the accumulated experience of the authors. I learned a lot from it, and I'm sure many others will too.

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Letter

A modest proposal

While reviewing *Computer Methods for Macromolecular Sequence Analysis* [this issue], I was struck by how difficult it seems to be for specialists in bioinformatics to give clear and authoritative advice to their experimental colleagues about the best methods to use for data analysis.

I suggest that this difficulty arises from two general problems. The first is the lack of agreed benchmarks or standards for program performance. Russell Doolittle, the editor of the above volume, has tried to fill this gap; several years ago he put considerable thought and effort into proposing defined benchmarks for the major problem of identifying proteins by family in long DNA sequences. It is to the discredit of the rest of the field that, as this new volume shows, his proposal has been lost without trace and not replaced by anything comparable. A significant exception is the 'CASP' (critical assessment of methods for protein structure prediction) series of exercises in blind secondary- and tertiary-structure prediction, but the format of these exercises could not easily be extended to other areas of sequence analysis.

The second problem can be illustrated by quoting articles from this volume and its predecessor, which both compare methods for sequence-database searching. In Volume 183 (1990), Pearson wrote 'searches with FASTA compare favourably with results using NWS-based programs that are 100 times slower.' In the corresponding chapter of Volume 226 (1996) he says 'For full-length query sequences, the Smith–Waterman algorithm [a specific NWS algorithm]...performs significantly better than FASTA', and he quotes a speed ratio of about 10:1. The Smith–Waterman algorithm pre-dates Volume 183 by nine years, and it is evident that what is being compared here are not pairs of methods or algorithms as such, but only pairs of *specific implementations* of different algorithms. If the objective is really to compare algorithms, then care has to be taken to see that they are all presented in the best light; if the objective is to provide practical advice to potential users, then, in addition, attention ought to be given to implementation issues such as programming efficiency and the stage of development of the software.

It seems to me that this is clearly not a practicable proposition, particularly in a field changing as fast as bioinformatics, and I suggest that we instead take a leaf from the book of the organic chemistry community, who constructed the library of guaranteed methods called Organic Syntheses in the early part of this century. I propose that we should have an annual publication of validated procedures in bioinformatics. The developer of any method could devise his own benchmark and propose it to the Editor – for example: 'here is a method to identify 70% of all members of a protein sequence family, when 75% of the residues are unmatched in the query sequence'; or 'here is a method to predict 80% of the four-residue turns in globular-protein sequences'. If the Editor thought the method to be of sufficient interest and generality, the author would prepare a complete and explicit recipe, with examples, which might be based on an algorithm, a running program or a networked service. The recipe would be passed to one or two independent labs for checking by explicit repetition and by the generation of new examples. Signed additions and other comments from the validating laboratories would form part of the final publication. Research workers in molecular biology could then have confidence that the methods they were using met a defined standard of performance, and the developers of new methods would have targets of performance that any new method would have to surpass.

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Workshop

Use of clinically relevant human–*scid*-mouse models in metastasis research

Improving cancer treatment so that the cure rate rises substantially is one of the most challenging and urgent problems facing clinical medicine in the Western world at the end of the twentieth century[1]. The incidence of several common forms of cancer, such as breast and colorectal cancer, is rising, and, despite progress in several areas of diagnosis and treatment, no overall increase in cure rate has been achieved. The failure of treatment can be attributed mainly to the lack of treatment options once metastases have developed[2].

Metastases are deposits of tumour cells, derived from the original primary tumour, that have spread to other sites in the body including, for example, the bone marrow, liver, lungs and brain. The formation of metastases is a multistep phenomenon involving the following stages: loosening of tumour cells from the original tumour; digestion of the surrounding connective tissue, including basement membranes; penetration of blood or lymphatic vessels; transport and survival in the circulation; adherence and extravasation from the blood or lymphatic vessel at a distant site; subsequent proliferation of the tumour cells at this new site; and eventually neoangiogenesis. As a consequence of this complex sequence of events, only a small proportion of cancer cells are able to form metastases[3].

The occurrence of multiple metastases in vital organs means that a surgical approach, as used for the treatment of many primary tumours, is not possible, and so metastases are

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treated with systemically administered chemotherapy. This approach is limited by the severe systemic side effects of the antineoplastic drugs and the development of (multi)drug resistance by the tumour cells.

Experimental approaches

Experimental studies of the processes involved in metastasis formation are difficult *in vitro* because it is impossible to model all the pathophysiological factors operative in a patient suffering from cancer. Some useful data may be gleaned from the examination of animals suffering from tumours in the same organ as a human tumour, but this approach is limited because the biological behaviour of an animal tumour does not necessarily correlate with that of the corresponding human tumour. Rodent breast cancer, for example, differs significantly in its biological behaviour from human breast cancer[4]. While it is possible, in principle, to transplant human tumours into animals, they will, of course, be rejected by the specific immune response of the host.

A variety of animal models has, therefore, been developed utilizing immunodeficient animals, such as nude mice or severe combined immunodeficient (*scid*) mice, in which transplanted tumours will not be rejected. Disadvantages of using nude mice include the residual elements of their immune systems including B and T cells, natural killer cells and macrophages. Previous studies using nude mice revealed that the majority of human cancer cells do not metastasize in these animals[5].

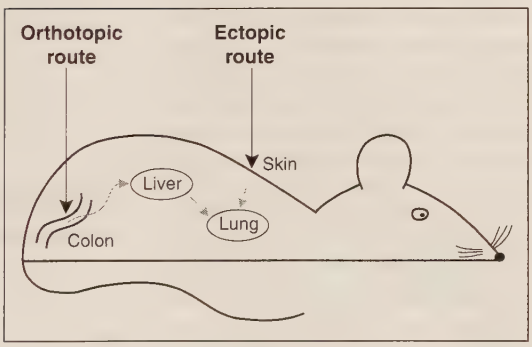

Figure 1

Different routes of tumour administration for the evaluation of experimental anticancer and antimetastatic treatments. The ectopic route involves the subcutaneous implantation of cancer cells, while the orthotopic route involves the implantation of cancer cells into the organ from which they were originally isolated. The cells then migrate (grey lines) to distant sites to form metastases.

A better, alternative, immunodeficient animal is the *scid* mouse[5], which is virtually free of B and T cells[6], and hence lacks a fully functional immune system. It could be argued that the influence of the immune system on the metastatic spread is, therefore, not adequately represented, but a comparison of carcinoma-infiltrated and non-carcinoma-infiltrated human lymph nodes in breast and colon cancer has revealed that the immunohistochemical profile (distribution of T- and B-cell subpopulations, macrophages, cell proliferation) of the two groups does not differ significantly. This finding indicates that the immune response *per se* is not involved to any great extent in the fight against breast and colon cancer once these cancers have reached the point where they are clinically detectable[7,8]. Nevertheless, this may not be the case where seeding of the tumour cells is occurring from the primary tumour and when cells are subsequently transported through the lymphatic or vascular system.

Routes of cell implantation

There are two routes commonly used for implanting tumour cells (Fig. 1): the ectopic route, in which cells are implanted subcutaneously, and the orthotopic route, in which cells are implanted into the organ in which the tumour first developed. The ectopic, subcutaneous, route for implanting cells can be criticized for using an abnormal site. Any spread from such a site may merely reflect seeding of cells via the vascular system, rather than metastasis *per se*, to reach the first capillary bed, the lung, which is one of the most prevalent sites for metastases to occur.

Another important factor in facilitating the development of metastases is a favourable microenvironment for the tumours. Indeed, attempts to mimic the *in vivo* situation have been made, in which extracellular matrix was combined with the tumour cells before they were implanted into the animal host[9,10]. The need for such elaborate preparations has been obviated in some cases by using orthotopic routes for implanting cells.

Identification of metastatic cells

Before any model of metastasis formation can be considered valid, it is necessary to distinguish those cells within the primary tumour that are capable of metastasis, for they are not all able to metastasize. This has recently been achieved by the use of the lectin from the Roman snail, *Helix pomatia* agglutinin (HPA). Lectins are sugar-binding proteins, and HPA binds to N-acetylgalactosamine. Although the binding partners of HPA in tumours have not yet been identified, its binding is indicative of metastasis formation and a poor prognosis in breast, colon, gastric, oesophageal and prostate cancers. Screening human colon and breast-cancer cell lines for HPA binding has revealed that only a few cell lines are HPA positive, and that these metastasize if transplanted into *scid* mice, while those that are HPA negative either do not metastasize or metastasize with a lower frequency[11,12]. Comparison of the expression of carbohydrate residues, as revealed by lectin histochemistry, in colon and breast cancer cell lines grown *in vitro* and *in vivo* in *scid* mice[13], has indicated that there is heterogeneity between similar tumours, both *in vivo* and *in vitro*. Thus, to maximize the effectiveness of treatment, therapies directed against more than one antigen should be more successful.

While metastases develop within three weeks in our model system (HT29 colon-cancer cells in *scid* mice), T47D or MCF7 breast cancer cell lines take up to nine months to metastasize in *scid* mice. This is a disadvantage for drug-evaluation purposes, although it does reflect the clinical situation, where the late occurrence of metastases is well known in breast cancer. For colorectal carcinoma, a five-year recurrence-free survival time generally indicates that the patient is cured, but it has been estimated by one group that this time should be as long as 19 years for breast cancer[14]. By using oestrogen-releasing pellets in experimental models, it is possible to accelerate tumour growth (Schumacher, U. and Adam, E., unpublished) and reduce the time periods. This allows the evaluation of new diagnostic and therapeutic strategies in a practical time span.

Applications and future prospects

The applications of these model systems in therapeutic development are considerable (Table 1). For example, HT29 colon-cancer cells are positive for the carcinoembryonic antigen (CEA). This antigen is the focus of recent work concerning the antibody-directed

Table 1. The use of *scid* mice in various recent experimental treatments of different cancers in which an increase in survival time has been demonstrated

Human cancer cell line implanted into *scid* mice	Therapy	Ref.
Chemotherapy		
Small-cell lung carcinoma	Cisplatin Mitomycin C	18
COL-2-JCK colon cancer	5-Fluorouracil	19
PRF/PLC/5 hepatoma cells	Vinblastin Doxorubicin Cisplatin	20
Small-cell lung carcinoma	Doxorubicin in liposome form	21
PC-3ML prostate carcinoma	Taxol	22
Antibody-mediated		
B-cell-precursor leukaemia	Anti-CD19–pokeweed antiviral protein	23
Hodgkin's lymphoma	Anti-CD30–ricin	24
Large-cell lymphoma JB6 cell line	Anti-CD30–saporin	25
SMMU-1 and -2 melanoma	Anti-CD44	26
Jurkat neoplastic T cells	Anti-T-cell (CD3)	27
Gene		
B-78-H1 melanoma cells	IL-6–sIL-6R gene therapy	28
Other		
M24met (melanoma)	Anti-(epidermal growth factor)	29
PC-3ML-B2 prostate carcinoma	Liarozole fumarate 13-*cis*-Retinoic acid	30
M24met (melanoma)	Disintegrin	31
MT539MG glioma	Herpes simplex viruses	32

Abbreviation: *scid*, severe combined immunodeficient; IL, interleukin.

enzyme–prodrug therapy (ADEPT) concept[15]. Tumour cells are targeted by an enzyme-conjugated antibody directed against the CEA. After the unbound antibody–enzyme conjugate has been cleared from the circulation, a nontoxic prodrug, which is enzymatically converted into the active cytotoxic drug on the tumour cell surface, is administered in order to kill the tumour cells[16].

Another classical approach, conventional chemotherapeutic regimes, can be evaluated in these systems. There has recently been a revival in the use of 5-fluorouracil (5FU) for the adjuvant chemotherapy of colorectal carcinoma[17]. Systemic administration of 5FU in our HT29 colon-cancer–*scid*-mouse model considerably reduces tumour weight (unpublished results), and so the effect of 5FU on the metastatic spread of tumours can be fully evaluated.

Our human–*scid*-mouse metastasizing-tumour model system therefore offers great potential for the testing and evaluation of new treatment strategies for metastasizing cancer, using standard chemotherapy drugs as well as novel treatment strategies.

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Microbiology of deep-sea hydrothermal vents

Daniel Prieur

Before the discovery of deep-sea hydrothermal vents in 1977, the deep sea was perceived as a cold, dark, high-pressure and nutrient-poor environment inhabited by psychrophilic, oligotrophic and barotolerant-to-barophilic microbial communities. By contrast, deep-sea-vent areas are warm-to-hot and inhabited by animal communities whose density may reach 50 kg m^{-2}. Invertebrates living in these warm biotopes are in endosymbiotic relationships with autotrophic sulphur-oxidizing bacteria. In the hot areas of the ecosystem, temperatures often reach 350°C, and precipitation produces mineral structures (black smokers) that contain thermophilic microorganisms.

The deep sea is currently defined as those areas of the oceans situated below a depth of 1000 m, and it has been estimated that this particular environment represents 62% of the total biosphere[1]. The deep sea is influenced by three main parameters: low temperature, high hydrostatic pressure and low nutrient concentration. This last feature is the consequence of the total darkness of the deep oceans, where, as a result, photosynthesis does not occur. Consequently, deep-sea animal communities have a low density and depend on the sinking from the euphotic zone of material that has escaped degradation in the upper layers of the oceans[1]. Thus the discovery of deep-sea hydrothermal vents and their luxuriant animal communities was certainly the most important event of the last 20 years for marine biologists[2]. For the first time, a productive ecosystem almost independent of solar energy was discovered, although the sulphur oxidation carried out by bacterial symbionts requires oxygen that is a product of photosynthesis[3]. Also, within a short distance of the warm-water communities, hydrothermal fluids with temperatures sometimes exceeding 350°C were observed, venting from mineral structures inhabited by hyperthermophilic microorganisms[4]. Thus within a rather small area (a vent site often does not exceed a few hundred square metres), there are communities that are very diverse metabolically, physiologically and taxonomically.

Presentation of deep-sea hydrothermal vents

Hydrothermal vents have been discovered in many tectonically active areas of the ocean floor, at depths from 800 to 3500 m and in both the Atlantic and Pacific oceans. In these regions, seawater penetrates through cracks in the sea floor, possibly down to several kilometers. This seawater is heated when it approaches the magma chamber, and leaches minerals from the magmatic basalts[5]. After a complex circulation, the hydrothermal fluids are emitted into the cold (2°C), oxygenated deep-ocean water. These hydrothermal fluids are acidic, reduced and enriched with chemicals including heavy metals, methane and hydrogen sulphide. If the fluids mix with cold seawater just before emission, their temperatures are moderate (10–30°C). If no mixing occurs, the fluids vent at temperatures of 350°C and above, remaining liquid because of the hydrostatic pressure (20 MPa at a depth of 2000 m). The mixing of hot hydrothermal fluids and cold seawater produces mineral precipitations and this causes mineral structures to form, sometimes 10–15 m in height, the 'smokers' or 'chimneys', from which fluids and particles vent at speeds of 0.7–2.4 m s^{-1} (Ref. 6).

Symbiotic life

The primary consumers thriving in deep-sea-vent areas are invertebrates that belong to new taxa[7], mostly vestimentifera, bivalves, gastropods, annelids or shrimps, depending on the oceanic region concerned. While the trophic diet of the shrimps (which swarm around the Mid-Atlantic-Ridge chimneys) and the annelids (which live on the outer wall of Eastern-Pacific-Rise chimneys) is not totally established, it has been clearly demonstrated that vestimentifera and molluscs live in close associations with endosymbionts. This symbiosis is particularly efficient in the case of the giant tube worm *Riftia pachyptila*, a vestimentiferan

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PII: S0167-7799(97)01052-4

that has no digestive tract but rather a special organ, the trophosome, full of monospecific endosymbionts[8]. In the bivalves (e.g. *Calyptogena magnifica*, *Bathymodiolus thermophilus*) and the gastropods (e.g. *Alviniconcha hessleri*), endosymbionts are located within the gill tissues, but these invertebrates do have a more-or-less functional digestive tract that enables them to feed temporarily on particles such as bacterial clumps[9].

Although these endosymbionts have escaped cultivation in the laboratory, they have been characterized by biochemical and molecular analysis. All are autotrophic Eubacteria that obtain energy from the oxidation of sulphides (methane oxidation has also been reported for symbionts of certain *Bathymodiolus*-like species) and fix carbon dioxide through the Calvin–Benson cycle[10,11]. They belong to the gamma subdivision of Proteobacteria that also encompasses nonsymbiotic sulphur oxidizers such as *Thiobacillus* and *Thiomicrospira*.

One important question is that of the transmission of symbionts between generations. In the case of vestimentifera, transmission seems to occur environmentally[12], but, for several bivalves, some arguments in favour of vertical transmission have been reported[13]. Several aspects of the process remain unclear. Symbionts that are thought to be acquired from the environment have never been detected out of their hosts, and putative vertically transmitted symbionts have been found only in the ovaria, not in the oocytes[13].

Shrimps and annelids do not have endosymbionts, but instead have epibionts, whose function has not been totally established. In shrimps, epibionts are located on parts of the mouth, but they have also been found on the outer walls of hydrothermal chimneys[14]. For annelids (e.g. *Alvinella pompejana*, *Alvinella caudata*), the epibiosis is very complex. The most-abundant forms are bacterial filaments, belonging to the epsilon subgroup of Proteobacteria[15], regularly inserted into certain specific regions of the worm's epidermis[16], but this epibiosis is made up of various morphological (rods, cocci, prosthecated cells) and metabolic (sulphur oxidizers, sulphate reducers, nitrifiers, denitrifiers, etc.) types[17].

Nonsymbiotic mesophiles

Mesophilic bacteria from mats, inert surfaces and water samples have been described[9]. Dense bacterial mats have mostly been observed in the Guaymas Basin, where they lie over the sediment through which the hydrothermal fluids percolate. They may reach several centimetres in thickness and consist of large filaments of *Beggiatoa* and *Thiotrix*[18]. Free-living sulphur oxidizers have been isolated and several novel species described (e.g. *Thiomicrospira crunogena*, *Thiobacillus hydrothermalis*)[19,20]. Also, a variety of heterotrophs (hyphomicrobiaceae, sulphate reducers, heterotrophic nitrifiers, manganese oxidizers) have been isolated and described[9,21,22]. Because hydrothermal fluids are enriched with heavy metals, heavy-metal-resistant bacteria have been investigated and described. A significant number of bacteria resistant to copper, cadmium, zinc, silver and arsenate have been found associated with the annelid worms (*Alvinella*) that live on the outer wall of hydrothermal chimneys and are particularly exposed to the metal-enriched fluids. About 25% of the bacterial strains studied harboured plasmids of various sizes, some of these plasmids being involved in resistance to arsenite and arsenate[23].

Thermophiles and hyperthermophiles

Hydrothermal vents have been investigated for the presence of thermophiles (optimal growth temperature >60°C) and hyperthermophiles (optimal growth temperature >80°C) because of the presence of fluids with temperatures above 350°C. Most of the strains isolated and described were strict anaerobes (fermentative heterotrophs, sulphate reducers, methanogens) belonging to the domain Archaea[24]. As the gradient between the hot, reduced fluid and the cold, oxygenated deep-seawater is very steep, it was thought that space for thermophilic aerobes could not exist. However, *Thermus* and *Bacillus* strains have recently been isolated from certain porous mineral structures that occur frequently along the Mid-Atlantic Ridge[25,26]. No novel genus specific to the deep-sea vents has been reported, but the deep-sea isolates are novel species (e.g. *Pyrodictium abyssi*, *Archaeoglobus profundus*, *Pyrococcus abyssi*, *Thermococcus profundus*, *Methanococcus jannaschii*) of genera previously described in coastal geothermal areas[24]. Several species had even been found previously in coastal areas (e.g. *Staphylothermus marinus*, *Methanopyrus kandleri*) and probably have a ubiquitous distribution[24].

One hypothesis that could explain this apparent lack of taxonomic novelty is that deep-sea hyperthermophiles have been isolated using methods designed for shallow-water organisms. In particular, hydrostatic pressure (a major parameter of deep-sea environments) has not been used for performing enrichment cultures. However, several deep-sea hyperthermophiles isolated at atmospheric pressure have been exposed to *in situ* pressures[27], which showed that the growth rate of most was increased by pressure and that their optimal and maximal temperatures for growth increased by 3–4°C. Moreover, while the deep-sea psychrophiles (cold-loving organisms) have an optimal pressure for growth below the pressure corresponding to the depth of sample collection, the hyperthermophiles have an optimal pressure above their *in situ* pressure. For this reason, it has been suggested that black smokers could represent 'windows to a subsurface biosphere'[27].

Genetic elements from hyperthermophiles

A number of plasmids and viruses from terrestrial aerobic hyperthermophiles are known, particularly from *Sulfolobus* strains[28]. Plasmids from deep-sea hyperthermophiles were first found in deep-sea methanogens (*M. jannaschii* and the related strain AG-86), with sizes of 64, 18 and 20 Kb (Ref. 29). More recently, plasmids have also been found in deep-sea

Thermococcales (anaerobic heterotrophic Archaea), and the plasmid pGT5 from *Pyrococcus abyssi* strain GE5 has been fully described[30]. This is a 3.5 Kb multicopy cryptic plasmid that replicates by the rolling-circle mechanism. The only gene of known function on this plasmid encodes the Rep protein, which is involved in plasmid replication. However, this plasmid represents the first genetic element suitable for the construction of a cloning vector for *Thermococcales*, studies of whose genetics are presently limited by the lack of genetic tools[31]. The search for genetic markers and transformation systems is presently under way in several laboratories.

Biotechnological applications of deep-sea-vent microorganisms

As described above, deep-sea-vent microbial communities are highly diverse metabolically, physiologically and taxonomically[9]. They are exposed to particularly extreme conditions – temperature gradients, hydrostatic pressure and high levels of toxic compounds (particularly heavy metals). For these reasons, they show a a great, but largely unexplored, potential for biotechnological applications. Several research groups are screening deep-sea hyperthermophiles for thermostable enzymes (e.g. amylases, glucosidases, pullulanase, xylanase, protease, DNA polymerases), and have already obtained promising results[32]. In addition, a cloning vector based on a *P. abyssi* plasmid would represent an important step for the genetic engineering of hyperthermophiles because recombinant proteins expressed in mesophilic hosts do not always have the same properties as the original protein[33]. Mesophiles also represent interesting sources for novel molecules and have been particularly investigated for novel exopolysaccharides that could be involved in heavy-metal binding[34].

Conclusions and perspectives

Deep-sea hydrothermal vents are usually considered as extreme environments. However, despite these conditions, the microbial communities living there are unusually diverse. For basic microbiologists, the mechanisms involved in adaptation to these conditions represent a variety of research topics. For microbial ecologists, many fascinating problems remain, such as the mechanisms of symbiont transmission, hyperthermophile distribution and dispersion, genetic exchanges at high temperatures, upper temperature limit for life, etc. For taxonomists and evolutionists, novel organisms or novel 16S rRNA sequences will probably be discovered. For biotechnologists, all the properties suggested above may represent novel sources of valuable molecules or processes. Unfortunately, progress remains slow, because access to these deep-sea sites is difficult, expensive and limited to a small number of investigators. An increase in the research efforts, accompanied by improved accessibility of microorganism collections in both academic institutions and industry on the basis of cooperation agreements, is probably the key to the future.

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Some aspects of bioassay methods in natural-product research aimed at drug lead discovery

Per Claeson and Lars Bohlin

The objective of natural-product research aimed at drug lead discovery is to find unique bioactive molecules with improved biopharmaceutical properties or new modes of action. In order to discover such bioactive substances, different bioassays are used for detection, for guidance of the chemical isolation procedures and for preliminary biological characterization of new compounds. This article gives a brief update on current biological activities and discusses some essential, but occasionally neglected, aspects of bioassay methods. Some future perspectives on the use of bioassay methods in the drug-discovery process are also discussed.

Plants, microbes and animals of terrestrial and marine origin are known to produce molecules with great variation in their chemical structure and biological activity. Some of these molecules exhibit biological activities that make them indispensable tools in biomedical research and unique prototypes for the development of drugs. In order to discover new bioactive substances, different bioassays are employed. Generally, the bioassay may serve one of three main purposes. Initially, it may be used to detect the presence of (and possibly to quantify) bioactive compounds in extracts of, for example, plants or microbial broths, which are often very complex. Secondly, it may be used to guide the chemical-fractionation procedure that leads to the isolation of pure bioactive substances. Finally, it may be employed to obtain an introductory biological characterization of an isolated compound.

Normally, a combination of several bioassays is used in natural-products-based drug lead discovery programmes. In the initial biological screening of large numbers of samples, the assays must be designed to have a high capacity and to deliver rapid answers at relatively low costs. The secondary bioassays are used to determine which 'hits' from the initial screening are samples worthy of further investigation. These assays must be performed carefully, and typically consume considerable amounts of time, effort and money.

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In this article, 'natural-product research' is understood to mean 'research aimed at the discovery of new bioactive molecules from natural sources'. Aspects of the use of bioassays in advanced pharmacometric evaluation of already-established drugs are not considered. Excellent review articles and book chapters covering experimental and theoretical aspects of different bioassay methods used in natural-product research have appeared over the years[1–7]. These publications are extensive and highly recommended. The purpose of this article is to give a brief update of recent research in the field and to discuss some essential, but occasionally neglected, aspects of bioassay methods and their use in natural-product research.

Biological activities in focus today

Table 1 lists the number of publications during 1996 that reported on biologically active natural substances. The table is based on the 1996 index of *Natural Product Updates*, which reviews all the articles that appeared in more than 130 of the major journals in the area of natural products. It should be noted that the table lists the number of *publications* dealing with bioactive compounds and not the number of isolated compounds, which is considerably higher. It is obvious from Table 1 that a lot of time and effort are spent on finding new antimicrobial agents. Substances with cytotoxic and antitumour activities are also frequently reported. The third-largest group of publications deals with inhibitors of various enzymes. It is interesting to note that large therapeutic areas like analgesia, diabetes

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PII: S0167-7799(97)01051-2

and neuropharmacology receive very little attention. It is possible that research in these important fields is considered less rewarding, and so avoided, because of the necessity of using complex and cumbersome bioassays to detect these types of activity. Antimicrobial effects, which are the most frequently published biological activities, are, in contrast, fairly easy and fast to assay *in vitro*.

The number of publications is only a rough estimate of the amount of ongoing research in a certain area. A large volume of research conducted within pharmaceutical companies is certainly never published, so these numbers mainly reflect research emanating from academic institutions. However, many reports are joint publications between researchers affiliated to academia and pharmaceutical companies, which could indicate that some industrial research is being reported.

Table 2 shows a classification of the main types of bioassay using three categories. The first of these consists of those assays related to infectious diseases and cancer, and, as can be seen from Table 1, this is the most popular area of research. The second type of assay mainly consists of methods developed in experimental pharmacology. The model systems range from classical experiments in live animals, through assays in isolated organs and cells, down to specific radioligand-binding assays on the receptor level. The third main type of assay relies on biochemical assays of isolated enzymes that are relevant for different diseases. A classification like this obviously involves a certain amount of overlap. Many enzyme assays, such as the HIV-1 reverse-transcriptase assay[15], also clearly relate to infectious diseases and cancer. Obviously, assays for antifeedants, insecticides, molluscicides and pheromones, included in Table 1, fall outside this classification.

Table 1. Publications dealing with biologically active natural substances that appeared during 1996 in international journals

Biological activity	Number of publications
Analgesics	13
Anthelmintics	13
Antibacterial substances	176
Antidiabetic substances	7
Antifeedants	19
Antifungal substances	91
Anti-inflammatory substances	22
Antimalarial substances	13
Antimitotic agents	5
Antioxidants	10
Antitumour substances	79
Antiviral substances	36
Cardiovascular agents	25
Cytotoxic substances	158
Enzyme inhibitors	115
Hepatoprotective agents	9
Immunomodulators	29
Insecticides	10
Molluscicides	7
Muscle relaxants	8
Neuropharmacological agents	10
Pheromones	52
Plant-growth modulators	17
Platelet-aggregation inhibitors	18
Toxic substances	71
Miscellaneous[a]	134

[a]Reports on less frequent (<5 publications) biological activities (antipyretic, cariostatic, antiandrogenic, etc.).

High-throughput screening

> High-throughput screening (HTS) is the process by which large numbers of compounds can be tested, in an automated fashion, for activity as inhibitors (antagonists) or activators (agonists) of a particular biological target, such as a cell-surface receptor or a metabolic enzyme.[16]

The origin of this technology was in the screening of microorganisms in the 1950s for the discovery of new antibiotics. The screening of natural products declined in the 1970s as a result of the discouraging rediscovery of previously described compounds and the strong development of medicinal chemistry and drug design based on synthetically derived substances. As a result of the rapid progress in molecular biology in the past decade, many new targets for therapeutic intervention have been identified, leading to the development of new enzyme and receptor-binding assays that have made the rapid biological screening of large chemical libraries possible. In addition, combinatorial chemistry now makes it possible to create chemical libraries of millions of compounds for testing; however, a lack of chemical diversity may be a limiting factor in drug discovery based on this type of combinatorial library. The awareness that nature exhibits unique structure–activity relationships and a proven ability to produce diverse and novel chemical prototypes make natural-product libraries once more of great interest for drug discovery[17,18]. When using combinatorial chemistry for natural-products drug discovery, a combinatorial library is built up around the structural motif of a known bioactive natural product ('biasing element')[17] and the library then subjected to HTS, in the hope of identifying a molecule with improved properties compared with the original, natural, product.

Today, there is also a growing interest in using plant cell cultures to produce compounds that have not previously been isolated from intact plants. To increase the chemical diversity, several techniques have been developed to affect the metabolite production of the plant by altering the direction and extent of chemical expression[17].

Single-target specific assays and multi-target functional assays

In addition to the descriptive classification of bioassays given in Table 2, another principal division of the assays into two distinct categories should be made.

Table 2. Classification of the main types of bioassay used in drug lead discovery[a]

Category	Examples	Ref.
Chemotherapeutic assays related to infectious diseases and cancer	Antibacterial assay	8
	Antimalarial assay	9
	P388 leukaemia assay	10
Pharmacological assays: *in vivo*, isolated organs, intact cells and receptor binding	Antihypertensive activity in rats	7
	Smooth-muscle-relaxing activity in the isolated guinea-pig ileum	11
	Platelet-aggregation assay	12
	Benzodiazepine receptor binding	5
Biochemical (enzymatic) assays	Cyclooxygenase assay	13
	Angiotensin-converting-enzyme assay	14

[a]Most bioassays employed in drug lead discovery based on natural-product research are adapted from methods developed in biomedical scientific disciplines such as pharmacology, biochemistry and microbiology. A stringent classification of such diverse methods is difficult to achieve, but, for the purpose of an overview, a pragmatic classification may be used.

The first type may be termed 'single-target specific bioassays'. These assays are typically tests on isolated enzymes or of receptor binding. They are characterized by a high degree of specificity, and an observed effect may be directly assigned to a specific mechanism of action, for example inhibition of the enzyme or affinity for the receptor. An attractive feature of these mechanism-based assays is that the procedures can often be automated and adapted to HTS.

The second category of assays may be called 'multi-target functional bioassays', and includes assays on whole animals, isolated organs and intact cells. These assays are broader in scope, and an observed effect cannot be immediately ascribed to a specific mode of action. For example, an observed relaxation of the isolated guinea pig ileum can be due to interactions at the levels of the cell membrane receptors, second messengers, ion channels, or to other mechanisms. To clarify the exact target of action of a new agent, a detailed evaluation involving the use of pharmacological tools and highly specialized experimental protocols is needed. These assays are fine-tuned and time-consuming procedures that are not easily automated. The multi-target functional assays based on established cell lines are important exceptions to this, in the sense that they often readily lend themselves to automation and HTS.

A most important step when selecting which bioassay to use is to define the aim of the research. The single-target specific assays can be used to identify new members of known mechanistic classes, such as finding a novel ligand to a certain receptor. Entirely new targets for or mechanisms of action by a drug will not be identified using this approach, but new chemical entities with superior biopharmaceutical profiles may be found. To detect compounds that act on previously unknown, but potentially useful, biological targets for drug action, multi-target functional assays are appropriate. Both known and hitherto-unknown targets for drug action are by definition present in relevant multi-target functional bioassays. Of particular interest is, of course, the identification of these new potential targets for drug action (e.g. new types of ion channels or second messengers) with the help of specifically acting, newly discovered bioactive compounds. The main disadvantage of these multi-target functional assays, on the other hand, is that they are, in most cases, too complicated, cumbersome and time-consuming to accommodate the large numbers of samples encountered in HTS-based drug-discovery programmes.

In principle, a decision thus has to be made on whether to search for an agent that exerts its biological effect by an established mechanism of action but with a new chemical structure that can serve as a structural lead for drug development (structural novelty), or to search for an agent that may act by a new mechanism or on a new biological target (biological novelty). The search for chemical novelty appears to have a somewhat higher probability of success due to the power of HTS. Clearly, the search for biological novelty has more of the character of basic research, with the intellectually challenging goal of finding completely new targets for drug action, with possible future application.

Future perspectives

Several trends related to the use of bioassay methods in the drug-discovery process are discernible. Clearly, further development and refinement of the technology that forms the basis for HTS will continue. This includes, for example, the development of new and more sensitive methods, such as the fluorescence-based assay technologies[19], for the detection of biological responses *in vitro*. The throughput rate will be further increased by the replacement of the 'classical' 96-well microtitre plates by 384-well or even 864-well formats. At the same time, the whole HTS technology is being miniaturized in order to reduce the material, time and cost spent on each sample. A distinction is already made between 'conventional HTS' and 'ultra-HTS', the latter using nanotechnology[16,20,21].

The real novelty and therapeutic potential connected with finding completely new targets for drug action will probably lead to intensified research, aimed at automation and the consequent inclusion of more multi-target functional assays in HTS.

Research that leads to a deeper understanding of how the bioassay results obtained when testing complex mixtures of natural products should be interpreted needs further attention. Closely connected to this issue is the need for strategies for the efficient combination of different types of bioassays to obtain early bioactivity profiles of extracts or compounds, and thus enable the clear direction of further research. Huge amounts of data are generated from HTS and the subsequent bioassay-guided fractionation of extracts. An efficient strategy for handling bioassay data is perhaps one of the most decisive factors in a successful outcome of natural-products-based drug-discovery programmes. The ongoing intensive development in this area of bioinformatics, exemplified by the use of the COMPARE algorithm[22], will no doubt continue.

Another crucial factor that closely relates to bioassay is the quality of the natural-product library that constitutes its input. One way of obtaining a high-quality natural-product library is to emphasize the selection of samples to be tested. A careful selection of test materials, based on a critical analysis of available ecological, ethnomedical and chemotaxonomical data on the organisms that produce them, is likely to increase the probability of finding new bioactive molecules.

Acknowledgements

We thank R. J. Quinn (QPRI-Astra Laboratories, Griffith University, Australia) and M. Divers (Astra HTS Centre, Lund, Sweden) for drawing our attention to some recent developments in the field of HTS.

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focus

Affinity cell separations: problems and prospects

John Hubble

The use of biospecific interactions for the separation and recovery of biomolecules is widely established, but developments in affinity-based separations for cell fractionation have been slow. Many of the problems encountered result from applying techniques developed for molecular systems to cell separation with only minor modification to the conditions. This approach tends to ignore, or at least trivialize, the problems that arise from the heterogeneous nature of a cell suspension and the implications of cooperativity that can occur with multivalent interactions. This article considers how alternative protocols might be used to develop more attractive cell-separation processes.

The basic concept underlying any biospecific or affinity-based separation is the interaction of a biological binding molecule (usually a protein) with its complementary ligand. Conditions are chosen to take advantage of the selectivity of these interactions and their low dissociation constant, such that an immobilized ligand can be used to adsorb the desired component effectively from dilute solutions. Once bound, the adsorbed material can be washed to remove unbound species; dissociation is then induced to allow recovery of the purified product in a more concentrated form[1].

While selectivity or specificity is important, the magnitude of the dissociation constant for the interaction is also crucial. The dissociation constant (K_d) is defined in terms of the ratio of unbound to bound molecules, and thus the lower the dissociation constant, the higher the fraction of product that can be bound during the loading stage.

The significance of K_d in determining product losses during the washing stage also needs to be considered. As washing proceeds, the concentration of product in the liquid phase will be reduced along with the contaminants to be removed. This means that the equilibrium between bound and free product will be displaced and some product will desorb. The extent of desorption will be a function of the wash volume used and the dissociation constant for the interaction. Essentially, the lower the dissociation constant, the larger the wash volume that can be employed for a given level of product loss. Hence higher purification factors should be possible with interactions that have a lower dissociation constant[2].

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At the elution stage, the definition of an ideal dissociation constant changes. At this point, the binding equilibrium must be displaced to allow the release of adsorbed product. If the original dissociation constant is too low, this will be difficult to achieve without the use of conditions that might damage the product.

Taken together, these factors mean that the ideal dissociation constant for a given separation will be a compromise between the requirements for the loading and washing stages and those for effective elution. These general considerations would be expected to apply to any affinity recovery system; however, when multivalent interactions can occur (as in cell separations, where contact will be made over an area containing a number of ligands and receptors) it is no longer possible to quantify adsorption in terms of a simple intrinsic dissociation constant.

In addition to complexities arising from multivalent interactions, there are a number of physical considerations that complicate the design of affinity cell separations. In a review of affinity chromatography of cells published in 1980, Sharma and Mahendroo[3] identified a number of problems that needed to be overcome if effective separations were to be developed. These included steric entrapment of particulate cells in packed agarose beds, the use of sugars to disrupt cells bound to immobilized lectins without the use of mechanical aids and the prevention of binding by agitation during the loading stage.

These experimental problems with traditional methods led to the development and rapid expansion of techniques based on immunomagnetic separations. In such separations, biospecific interactions are used to couple magnetic particles to the target cell population, which can subsequently be recovered using a magnetic

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PII: S0167-7799(97)01057-3

field. This technique is both simple and effective to use, and has been widely employed in diagnostic applications and small-scale separations[4]. Magnetic cell separation (MACS) has been successfully scaled up for the preparation of specific cell lines, but the technique can be expensive and removal of the bound beads from the target cells after separation can be difficult[5]. Given these limitations, it is worth considering the potential of alternative approaches to affinity cell separation in the light of recent developments in membrane- and column-based methodologies.

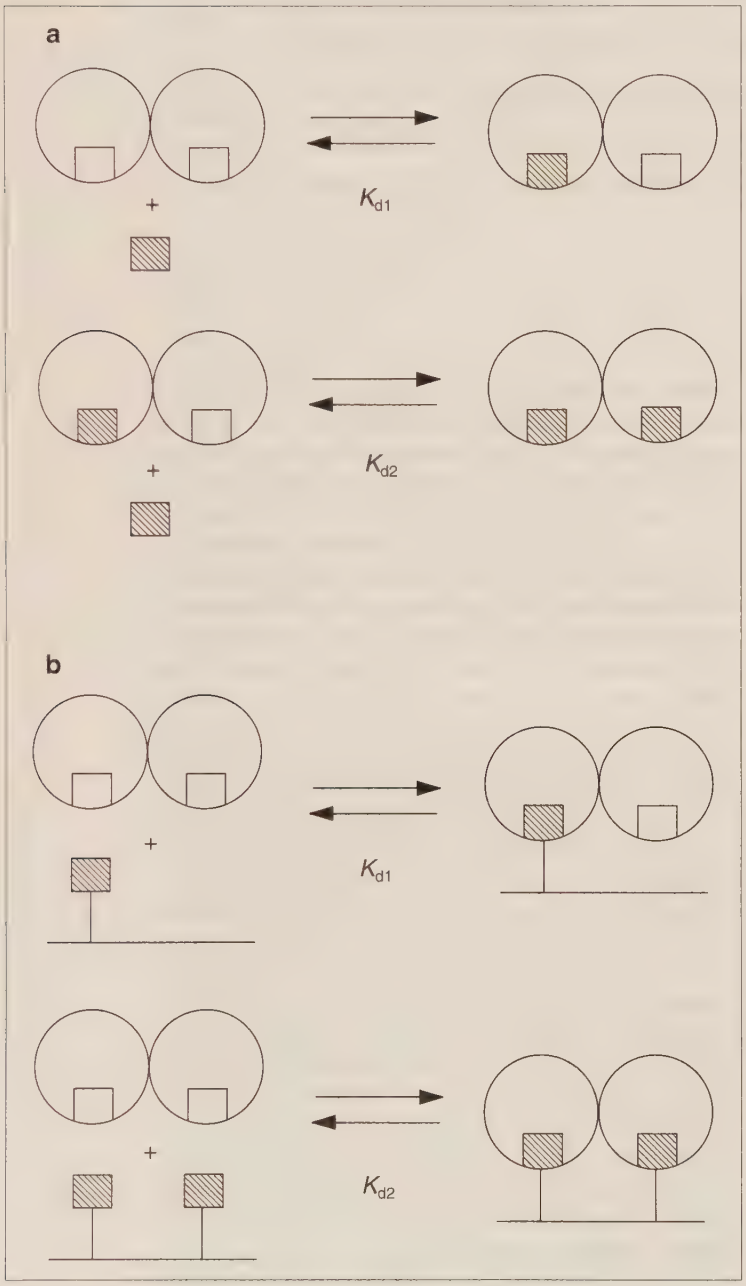

Figure 1

(**a**) Interaction of a soluble ligand with a divalent enzyme showing intrinsic positive cooperativity (i.e. dissociation constant $K_{d1} > K_{d2}$). (**b**) Interaction of an immobilized ligand with a divalent enzyme showing no intrinsic cooperativity. Surface cooperativity leads to enhanced binding (i.e. dissociation constant $K_{d1} > K_{d2}$).

Fundamental considerations

While the majority of molecular interactions used in affinity-based separations are monovalent, there are a number of enzymes that are dimeric or tetrameric. This introduces the possibility of two forms of binding cooperativity. The first arises from the intrinsic cooperative properties of some multimeric enzymes[6]. In a multivalent enzyme showing positive cooperativity, the dissociation constant for a given site will decrease with the level of occupancy of the other sites on the molecule (Fig. 1a). Thus the apparent dissociation constant will be a function of the number of sites occupied and not a simple constant. In the case of an intrinsically cooperative enzyme, the level of site occupancy will be a function of both the ligand spacing on the immobilized-ligand support surface and the free ligand concentration[7,8]. It has been shown in these cases that the presence of free ligand can enhance binding under some conditions by increasing site occupancy so that the apparent dissociation constant is reduced.

The second form of binding cooperativity arises from the proximity of ligands on the support and may affect multisite enzymes that do not show intrinsic cooperativity[9]. In situations where the ligand distribution includes clusters of ligands in close proximity, it is possible for a multimeric enzyme to form multiple interactions with the surface, again leading to an apparent reduction in the observed dissociation constant[10] (Fig. 1b).

When the adsorption of cells or particles to surfaces is examined, it is clear that the potential of multiple interactions is significantly greater than that of molecular systems. For cell separations, cell-size and geometric considerations can be used, together with the ligand and receptor densities, to determine the maximum number of interactions (n) that can occur between a cell and an affinity surface[11] (Fig. 2). A typical ligand and receptor density range of 10^{10}–10^{12} cm^{-2} and a contact area range of between 10^{-10}–10^{-8} cm^2 suggest that n could have values between 1 and 10 000.

The dependence of fractional binding on the number of possible interactions can be derived in a similar manner to the fractional saturation of sites in a multivalent enzyme (as in the case of the Adair equation[12]) to give[13]:

$$Y = 1 - \frac{K}{K + [\mathrm{R}](\Sigma_{i=1}^{n} iK'^{i-1})} = \frac{[\mathrm{R}]}{K\frac{(K'-1)}{(K'^{n}-1)} + [\mathrm{R}]} \qquad (1)$$

where Y is the fraction of ligand bound, R is the receptor, L is the ligand, [ ] are used to denote concentration of the enclosed species, K is the dissociation constant for the first interaction between a ligand and receptor, K' is a surface-association constant given by $[\mathrm{LR}_i]/[\mathrm{LR}_{i-1}]$ (i.e. the ratio of the ligand–receptor complexes held through i interactions to the concentration of those held through i–1 interactions) and n is the maximum number of possible interactions.

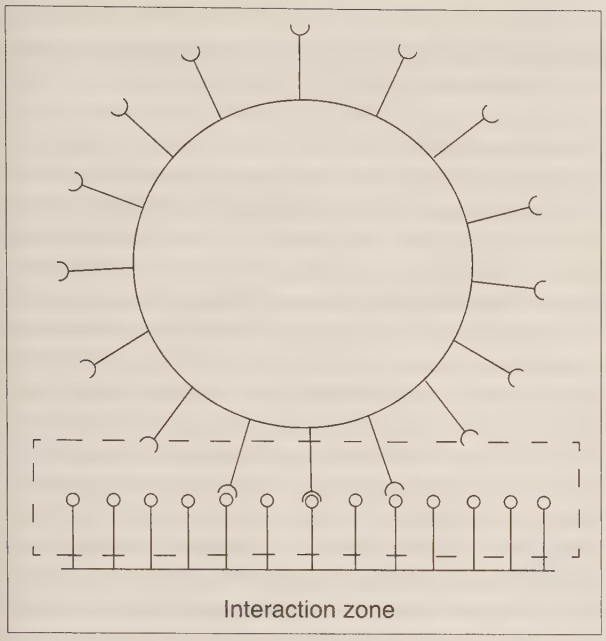

Figure 2
Ligand–receptor-mediated binding of a cell to a surface. Broken line shows the limits imposed on the number of interactions.

The effect of multivalency is thus to modify the apparent dissociation constant by the term, $(K'-1)/(K'^{n}-1)$, and so, for K' values greater than one, fractional binding will increase with the number of possible interactions as the apparent value of the dissociation constant decreases. In the case of a monovalent interaction, $n = 1$ and Eqn 1 reduces to a simple Langmuir form:

$$Y = \frac{[R]}{K + [R]} \qquad (2)$$

These relationships predict that surface ligand density will determine the apparent dissociation constant for adsorption through its effect on n. In addition, surface concentration also determines the average spacing between adjacent ligands and the probability of localized ligand 'clusters'. Both of these effects will influence the value of K' and hence will again modify the apparent dissociation constant. The experimental significance of multivalency is shown in a report by Adler *et al.*[14], who, studying the effectiveness of binding inhibitors, found an increase of five orders of magnitude when comparing a protein conjugate containing 39 saccharide moieties with the monomeric saccharide inhibitor.

Clearly, the consequences of multiple interactions have significant implications for the design of affinity separations, and these are best considered in terms of the individual process stages.

Loading

With heterogeneous systems comprising a suspension of discrete particles that are acting as ligand or receptor clusters, the concept of a volume-averaged ligand and receptor concentration has little meaning. Initial contact between 'adsorbate' and 'adsorbent' will be largely dictated by gross physical effects rather than diffusivity at the molecular level. This means that the standard kinetic assessment based on the measurement of an 'on' constant, determined mainly by the diffusion coefficient of a molecule, and an 'off' constant, determined by the nature of the interaction, is inappropriate.

For cell separations, the initial stages of loading, where contact is engineered between particle and adsorbent, will be dictated by particle mechanics rather than molecular kinetics, and the unit design should reflect this. Once contact has been achieved, the biological interactions that form will determine the strength of attachment or retention. As described above, binding can be quantified in terms of the number of possible interactions. Given that each interaction can be assigned a bond strength, the force required to remove the cell from the surface can be quantified[15].

While the equations developed above consider the effects of multiple interactions on binding equilibria, it is also possible for the kinetics of loading to result in a steady-state situation that is dependent on the initial conditions used. Even if the theoretical maximum number of interactions is determined by geometrical and binding considerations, there is no guarantee that this maximum will be achieved (Fig. 3). Unless the ligand and receptor distribution are both uniform over the contact area, it is probable that the number of bonds that form will be dependent on the orientation

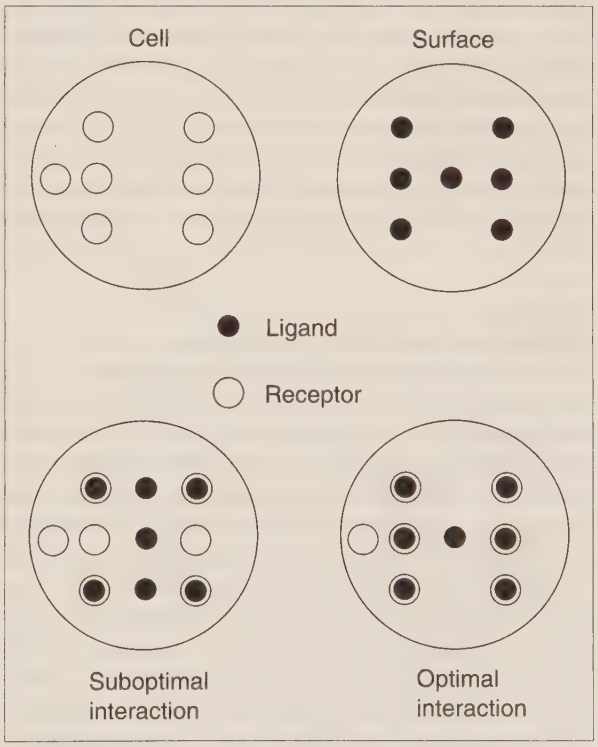

Figure 3
Ligand–receptor-mediated interaction of a cell with a surface, showing the importance of orientation in determining the number of interacting ligand–receptor pairs. Note that even in the optimal orientation it cannot be assumed that the theoretical number of interactions will be reached.

of the initial contact and the rate at which interactions subsequently occur. The situation might be regarded as analogous to annealing, where the ultimate crystal structure of a metal is dependent on the rate at which it is cooled from a molten state. There is certainly a large amount of data showing that multivalent interactions are stabilized over time. For example, the ability of a monovalent ligand to displace a polyvalent complex based on human serum albumin that had been bound to a human cell line via an antibody-mediated interaction was found to be strongly dependent on the prior incubation time. Preincubation times of as little as five minutes resulted in 25–30% of the complex remaining bound to cells after the introduction of competing monomer[16–18]. In many cases, this results from cellular responses, where the antibody is able to migrate through the cell membrane and accumulate in the vicinity of the bound complex so that the number of bonds increases with time[19]. However, unpublished results obtained in our laboratory with both artificial cell mimics and yeasts show that there can also be a time-dependent stabilization effect in systems where such surface migration of binding molecules is not possible. This suggests that it is also possible for the whole complex to reorientate with respect to the surface over a period of time.

Given that the final number of bonds between cell and surface may be determined by the sequence as well as the extent of the reaction, it is probable that the attachment force can be controlled by the loading conditions as well as the ligand density used. For example, it is possible that the loading stage can be moderated by the use of temperature or the presence of competitive monovalent inhibitors to give the ultimate binding strength required. Indeed, a very close analogy to annealing could be obtained by the cells physically contacting the surface in a high concentration of inhibitor, which is then progressively reduced as binding proceeds.

Washing

If well designed, the contacting stage will ensure that only cells of a similar size and density settle or are deposited on the adsorbent surface. The interactions that subsequently form will determine the strength of attachment. The aim, as with any affinity system, is to choose conditions so that contaminants will not be able to interact with the support. This means that non-recognized cells will have weak attachments and will be easy to remove by the application of a small force. Thus, in a liquid system, the fluid shear can be adjusted so that cells having no biospecific interactions with the surface are removed. There are two significant differences between this and a molecular separation. Firstly, it is the applied force that removes contaminants to improve purity, not the displacement of a weak equilibrium or simple replacement of the contact fluid with a clean buffer. Therefore the flow characteristics are more important than the wash-fluid composition. Secondly, as washing is not based on equilibrium displacement, it will not necessarily be accompanied by the loss of product cells, and the trade-off between purity and recovery encountered in molecular systems can be avoided.

Elution/recovery

In most affinity chromatography systems, there are two main approaches to inducing elution. Most commonly, the process conditions are changed so that the interaction is disrupted; typically salt, pH or temperature are used for this purpose. The requirement is for conditions that induce a sufficiently large structural change to promote desorption, without causing denaturation or product damage. As stated earlier, the feasibility of this is a function of K_d such that if K_d is too low, excessively harsh conditions may be required. A more elegant approach to inducing desorption is offered by the use of biospecific eluents, where competing free ligand is used to displace the binding equilibrium. The problems here are that the cost of the eluting agent may be prohibitive and that the rate of desorption is often low as it will be dictated by the 'off' constant of the interaction.

In cell-based systems, an additional approach is possible, namely to apply an external force in excess of the attachment force holding the cell to the surface[20]. In practice, this may be used in conjunction with the other methods to reduce the force required. Using an extension to the approach taken to describe fractional binding in multivalent systems, the effect of applied force on the fraction of attached cells can be derived[21].

The total number of bound cells (Cb_{tot}) is given by:

$$Cb_{tot} = \Sigma_{i=1}^{n} K'^{i-1} \frac{[C][L]}{K_d} \qquad (3)$$

where (C) is the suspended-cell concentration. The bound cells removed by an applied force (Cb) is given by:

$$Cb = \Sigma_{i=1}^{nf} K'^{i-1} \frac{[C][L]}{K_d} \qquad (4)$$

The number of bonds required to maintain attachment, nf, can be calculated from estimates of the applied force and the individual bond strength using relationships such as that given by Hammer and Lauffenburger[22]. The fraction of attached cells (F) remaining on the surface can be expressed as:

$$F = \frac{Cb_{tot} - Cb}{Cb_{tot}} \qquad (5)$$

Substituting for Cb_{tot} and Cb using Eqns 3 and 4 and simplifying gives:

$$F = \frac{\Sigma_{i=1}^{n} K'^{i-1} - \Sigma_{i=1}^{i=nf} K'^{i-1}}{\Sigma_{i=1}^{n} K'^{i-1}} = \frac{K'^{n} - K'^{nf}}{K'^{n} - 1} \qquad (6)$$

If the strengths of all bonds are considered as equal, n can be determined from the ratio of the applied force to the force required to break one bond. Thus Eqn 6 can be used to calculate the extent of detachment that will result from a given applied force.

The term $[C][L]/K_d$, which cancels out of the equations, can be assumed to be independent of applied force during detachment as it will define only the last, monovalent, interaction between cell and surface. This expression suggests that, as might be expected, under detachment conditions the effect of applied shear will be independent of the suspended (i.e. unattached) cell concentration.

In any experimental system, some applied force will be necessary to physically remove the cell from the surface, even if all of the biospecific interactions have dissociated or been blocked (in this situation, cell recovery would be analogous to resuspending a settled suspension). The amount of force required above this minimum will depend on the extent to which the molecular interactions can be perturbed. As with molecular separations, the most feasible approach is to use nonspecific eluents to change conditions so that binding is no longer favourable. The constraint here is the extent to which cell viability is compromised by the use of elevated salt concentrations or pH shifts. Although the cooperative effects of multivalent interactions will significantly reduce the apparent dissociation constant and compromise the use of biospecific eluents, nonspecific eluents that modify the intrinsic dissociation constant may be effective in many cases.

In the case of biospecific elution, the concentration of a monovalent competitor would need to be determined with respect to the apparent dissociation constant of the multivalent interaction. In systems where n can be large, this would lead to concentrations that are not physically achievable. The situation can be improved by the use of multivalent competitive inhibitors[23]. Although their use has not been reported in affinity separations, studies conducted on cell–cell interactions have shown that multivalent inhibitors can be many times as effective as an equivalent monovalent species. This effect has been demonstrated for the inhibition of L-selectin by glycan sulphates, where the affinity of the interaction increases with molecular weight (and hence valency)[24]. The specificity of these effects is shown by the work of Biessen *et al.*[25], who found that a cluster synthesized with a spacer between ligand and backbone had 2000 times higher affinity than a similar complex without the spacer. However, although these findings have significant therapeutic implications, the cost of such preparations would make them unattractive for use in cell-separation systems. In addition, as with molecular separations, the kinetics of biospecific displacement are likely to be very slow.

Considerations for effective separator design

There are two central factors that emerge from the assessment of the fundamentals of cell–surface interactions – the importance of applied force and the significance of multivalency. These suggest that protocols based on the use of packed beds and biospecific elution are unlikely to be successful.

Taking the physical design of the contactor first, the fundamental considerations dictate a geometry that allows for effective contact of the cells with the surface and has the potential to control the force subsequently applied to attached cells by varying the fluid shear at the surface. It is immediately apparent that a fixed-bed adsorber configuration will not satisfy these requirements. In addition to the problems of steric entrapment identified by Sharma and Mahendroo[3], the compressible nature of agarose beads coupled with the pressure-drop flow relationships inherent in fixed-bed operation will place unacceptable limits on the displacement force that can be applied to attached cells. However, recent developments in the area of molecular adsorption include the use of expanded adsorbent operated at high superficial fluid velocities in an up-flow configuration [see R. Hjorth, last issue]. This approach is attractive, as it allows the column to be fed with relatively crude cell homogenates that would block a column operated in a packed configuration, and allows adsorption to be employed earlier in a separation sequence[26]. In the case of cell separations, many of the problems of steric entrapment of cells would be alleviated by the more open structure of an expanded bed. Similarly, the greater range of flow rates allowed (particularly if dense nonporous beads are employed) means that attached cells can be exposed to a wider range of shear forces than would be possible in a packed bed.

An alternative approach, which offers a number of attractive possibilities, is the use of a membrane-based system. Membranes can function either solely as sieves or as combined sieve–adsorbers[27]. In many molecular separations, the potential benefits of the combined approach are compromised by fouling effects, which can lead to rapid changes in the apparent molecular weight cutoff. However, the membrane adsorber is particularly well suited for use in cell separations, where the potential to adjust the balance between transmembrane flux and fluid velocity parallel to the surface means that adsorption, washing and elution steps can be carried out under separate, individually optimized, conditions (Fig. 4).

In mode of operation, a membrane-based approach offers the closest analogy to the flow-cell geometries typically used in cell–surface detachment studies, and so flow conditions can easily be designed to match those identified in assay systems. In addition to this advantage, the ability to generate a convective flow normal to the membrane surface allows effective control of the contact/adsorption stage of the separation (Fig. 4a). For cell separations, the membrane pore size can be such that concentration polarization and fouling by macromolecules can be minimized, and while biocompatibility considerations may be important, the issue of mammalian cell stability has been addressed in the context of membrane bioreactor design[28].

The suitability of membrane separators for the washing and elution stages of separation also stems from the ease with which surface shear can be varied, and again there is a large amount of background information, in this case resulting from the use of cross-flow-induced shear to minimize membrane concentration polarization and fouling[29]. There are two additional factors

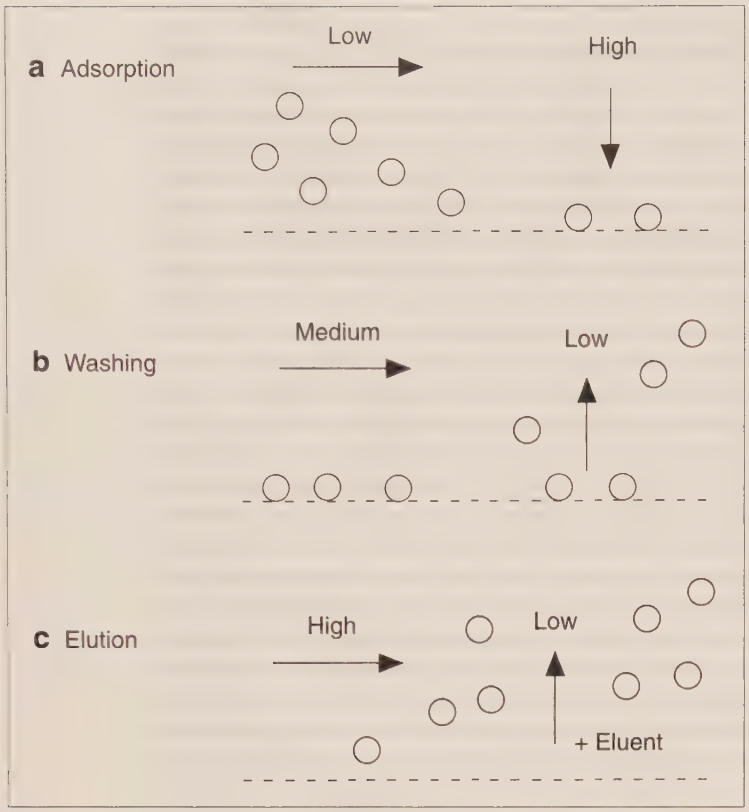

Figure 4

Schematic of a membrane-affinity surface used for cell separation. (**a**) Adsorption/loading: low cross-flow velocity is used to minimize shear-induced detachment, while a high transmembrane velocity is used to deposit cells on the surface (i.e. high concentration polarization). (**b**) Washing: medium cross-flow is used to generate sufficient surface shear to detach cells held solely by nonspecific interactions, while a low transmembrane velocity in the reverse direction (backflush) is used to minimize collision effects. (**c**) Elution: high cross-flow is used to generate sufficient shear to detach affinity-adsorbed cells. Low backflush allows the introduction of nonspecific eluent.

that increase the attractiveness of membrane separators. The first is the problem of nonspecific detachment of target cells during the washing stage. Even though it may be possible to wash with a shear rate below that needed to detach specifically bound cells, they can still be removed as a result of collisions with contaminant cells washed from the surface. The ability to use a membrane with either a continuous or periodic back flux offers the potential to move cells away from the surface after detachment so that the potential for collision-induced detachment is reduced (Fig. 4b). However, the second, and probably most significant, advantage offered by membrane-based cell separators is the potential they offer for the effective use of nonspecific eluents. In traditional fixed-bed configurations, the concentration of nonspecific eluents required is likely to be highly cytotoxic and, in the case of mammalian cells, to cause unacceptably large changes in osmotic pressure. However, in a membrane system this problem can be avoided because the permeable nature of the support surface allows very precise control of eluent delivery (Fig. 4c). An interesting example of this is given by Mandrusov *et al.*[30], who adsorbed cells onto membrane-immobilized ligand and then effected elution by allowing hydrochloric acid to diffuse through the membrane while generating a fluid flow across the membrane surface. The mass-transfer and dilution effects in this system are such that there will be a pH gradient from the membrane surface. While the acid concentration in the vicinity of the ligands is high enough to disrupt binding, the concentration to which detached cells are exposed is too low to cause significant damage.

These authors demonstrated the effectiveness of this approach by comparing three elution protocols: (1) pH 5 applied to the cell side of the membrane, which resulted in 30% viability of recovered cells; (2) pH 2 applied to the cell side of the membrane, which resulted in 10% viability of recovered cells; (3) pH 1 applied to the opposite face of the membrane, which resulted in greater than 60% viability of recovered cells. All three protocols were conducted under identical flow conditions and all gave total removal of cells from the surface.

Conclusions

Problems associated with the application of affinity techniques developed for use with molecular systems to cell-based separation have led to the successful development of novel alternative approaches based on magnetic-affinity beads. However, this technique is also not without its problems, and the difficulty of disrupting multivalent interactions described in this article may explain the difficulties encountered in separating cells from beads once the recovery is complete[5]. While few studies have directly focused on the scale-up of affinity-mediated cell fractionations based on the approaches used for molecular separations, there has been a continued improvement in our understanding of the nature and mechanics of the ligand–receptor-mediated interaction between cells and surfaces. This, coupled with recent developments in the areas of adsorption chromatography and membrane technology, suggests that many of the earlier perceived problems can now be overcome and that the design of competitive cell-separation processes should be achievable with existing downstream-processing technology.

This article has focused on a number of phenomena that are important in determining the extent and strength of attachment. In contrast to a typical analysis for soluble systems, ligand-density effects have been considered largely in terms of their effect on the number of possible interactions rather than in terms of concentrations. Similarly, the relationship between apparent dissociation constants for multivalent interactions and the intrinsic dissociation constant has been derived with respect to the number of interactions. The implication is that, above a critical value, increases in ligand density would be expected to have little influence on attachment, and this agrees with the observations of Ward *et al.*[31], who showed that, at high ligand densities, the critical tension required to peel a cell from a surface is independent of ligand density.

Once multiple bonds have formed, the theory presented suggests that the intrinsic dissociation constant has little direct influence on the stability of attachment. What is important is the speed at which these bonds can both form and break. It is interesting to note that the selectin molecules that mediate multivalent interactions between neutrophils and the walls of blood vessels are characterized by large dissociation constants and an abnormally high 'off' constant[32]. Taking a lesson from nature, it would appear that the ideal interaction to use in a cell separation should have a dissociation constant of the order of 10^{-4} M, probably two orders of magnitude higher than would be typical for a molecular-affinity separation.

Once the ligand–receptor interaction to be used has been identified and the maximum possible bond number determined, the critical factor for success will be the ability of the separator to allow the application of the optimal removal force during the washing and elution stages. At the current time, a membrane-based adsorber would appear to offer the most flexible approach to meeting these requirements.

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Aspergillus as a host for heterologous protein production: the problem of proteases

Johannes P. T. W. van den Hombergh, Peter J. I. van de Vondervoort, Laurence Fraissinet-Tachet and J. Visser

Homologous and heterologous protein production by filamentous fungi is often limited by the expression of proteases at high levels. By eliminating specific protease activities, protein production in *Aspergillus niger* can be improved considerably. Both classical mutagenesis and gene disruption techniques have yielded strains with reduced protease expression. Combinations of these mutations and disruptions result in a further reduction of protease activity. The coupling of efficient promoters to target genes allows their expression under conditions that repress the expression of several proteases, which further improves product yields. The strategies used have led to the development of a set of tester strains from which the appropriate genetic background for production can be selected.

Filamentous fungi belong to a diverse group of lower eukaryotic microorganisms, some of which are used in a variety of industrial processes including the production of fermented foods[1], primary metabolites such as organic acids and vitamins[2], and secondary metabolites (antibiotics, alkaloids, gibberellins)[3,4]. Moreover, the filamentous fungi are excellent producers of a broad spectrum of extracellular enzymes [amylases, proteases, (hemi-)cellulases, pectinases, catalases, lipases, phosphatases and glucose oxidase][2,5]. More recently, these fungi have been developed as host organisms for the production of foreign (heterologous) proteins[6–8]. The exploration of filamentous fungi was considered to be very attractive as they have a number of advantages compared with other expression systems. These advantages include: (1) the ability to produce and secrete exceptionally large amounts of proteins; (2) bacterial and yeast systems are sometimes unable to express certain proteins, especially eukaryotic proteins that require post-translational modifications such as glycosylation and disulfide bridge formation, whereas filamentous fungi have been found to express some of these proteins in the correctly folded and functional form (see Ref. 8 for a recent review); (3) several production processes involving filamentous fungi have GRAS (generally regarded as safe) approval, which allows food-grade applications; and (4) stable recombinants can be isolated, thus enabling controlled strain breeding.

Filamentous fungi of industrial interest include *Aspergillus* species such as *A. awamori*, *A. niger*, *A. oryzae* and *A. nidulans*, *Mucor* species (e.g. *M. miehie*) and *Trichoderma* species (e.g. *T. reesei*).

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The protease problem

Although several proteins have been successfully expressed in *Aspergillus*[8], the secreted yields of heterologous proteins rarely reach the gram-per-litre level of many homologous enzymes, thus indicating the need for strain improvement[6–8]. Improvements in secreted heterologous protein yields are often obtained by increasing the heterologous mRNA levels, provided sufficient associated regulatory proteins are also present, and/or by using promoters in combination with high copy numbers[6]. In some cases, the site of integration of the introduced genes has been found to have an influence on expression levels, and so increased expression levels may also be achieved by reducing negative integration effects[7]. In many cases, problems with heterologous protein production do not appear to be at the level of transcription but rather occur

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PII: S0167-7799(97)01020-2

within the secretory pathway or following secretion[8]. Therefore a better understanding of the protein-secretion pathway and the factors involved in the stability of the heterologous protein are crucial.

The strain-improvement programmes for the heterologous production of chymosin in *A. awamori* and *T. reesei* are excellent examples of how to produce efficient strains. These strains, which secrete chymosin at commercial levels, have been generated via mutagenesis, genetic recombination and genetic engineering[9,10]. An important step in achieving high expression yields, especially in *Aspergillus*, involved the reduction of protease activities[9]. The growing interest in using filamentous fungi as heterologous expression–secretion hosts for the production of proteins by recombinant DNA technology has brought this problem into focus, as heterologous proteins are often more prone to proteolysis than homologous proteins[11–13]. Proteolytic degradation by fungal proteases is still recognized as one of the major problems interfering with efficient heterologous protein production[6,7,8,14]. Bioprocessing steps such as downstream processing at low temperatures, the early separation of product and protease(s) or the use of protease inhibitors might reduce proteolysis. However, they will certainly not eliminate the problem because much of the degradation occurs *in vivo* during the production of the protein[8].

Tackling the protease problem

The spectrum of proteases in individual *Aspergillus* species can be very diverse and species-specific[12], indicating that the choice of the host strain is very important for optimal yields of heterologous proteins; in this article we will give a detailed description of the proteolytic spectrum of *A. niger*. The different approaches used to reduce proteolytic degradation of the expressed products can be transferred to other filamentous fungi to improve protein production, although this requires efforts to develop classical genetics for the system (J. P. T. W. van den Hombergh, PhD Thesis, Wageningen Agricultural University, 1996).

Analysis of the proteolytic spectrum in *A. niger* revealed that acid proteases predominate (Fig. 1). Four such extracellular proteases with acid pH optima [two aspartyl proteases, PEPA and PEPB, and two serine carboxypeptidases, PEPF (or CPDII) and PEPG (or CPDI)] have been purified from culture filtrates[15–21]. These extracellular proteases are probably involved in the hydrolysis of nutrients to be used as carbon, nitrogen and sulfur sources. *A. niger* is known for its strong acidification of culture media, which explains why acidic protease activities cause the major problems during homologous and heterologous protein expression. In other *Aspergillus* species, such as *A. nidulans*, neutral and alkaline proteases seem to play a more important role and acid proteases are weakly expressed (J. P. T. W. van den Hombergh, PhD Thesis). The genes encoding several of these proteases have been cloned[20,22,23] and a fifth protease gene, encoding an extracellular subtilisin-type serine protease, PEPD, has been recently cloned via PCR techniques based on conserved amino-acid sequences within subtilisins[24]. Furthermore, three proteases that are homologous to yeast vacuolar proteases have been cloned from *A. niger* – *pep*E a pepsin-type aspartyl endoprotease that is the homologue of the vacuolar *pep4* gene product in yeast[25], *pep*C, a subtilisin-type serine endoprotease[26] and *cpy*, a serine carboxypeptidase[27]. Based on the strong homologies with the yeast; proteins, the *A. niger* proteases are probably involved in nonspecific protein degradation and turnover, and in the activation of the precursors of several other vacuolar enzymes.

Disruption of interfering protease genes

As the major degradation-related problems in *A. niger* are caused by acid proteases, we started a disruption programme to construct strains in which one or several proteases were specifically eliminated. Previously, it was demonstrated by Berka *et al.* that disruption of the major extracellular acid protease PEPA in *A. awamori* reduced extracellular proteolytic activity to approximately 20% of that of the wild type[20]. Similar results were obtained upon disruption of the *A. niger pep*A gene[28]. We have now disrupted three different acid-protease genes in *A. niger* – *pep*A, *pep*B and *pep*E. The resulting single disruptants, Δ*pep*A, Δ*pep*B and Δ*pep*E, allowed analysis of the contribution of the individual proteases to the overall protease spectrum, using bovine serum albumin as a broad protease substrate. The PEPA and the PEPB proteases constitute 84% and 6% of the extracellular acidic proteolytic activity respectively, whereas the PEPE protease is responsible for 68% of the intracellular acidic protease activity. Furthermore, in the Δ*pep*E disruptant, intracellular serine endopeptidase and serine carboxypeptidase activities are also reduced. This may indicate that PEPE is involved in the cascade activation of some serine endopeptidase and serine carboxypeptidase activities in the vacuole, in a similar way to that found in yeast[29].

To establish the effects of the reduced protease activities on the stability of proteolytically susceptible proteins, we first used an *in vitro* degradation assay. In this assay the proteolytically susceptible *A. niger* PELB protein, a recently purified pectinolytic enzyme, is incubated in serial dilutions of a culture filtrate and the residual PELB activity determined and compared with the wild-type culture filtrates[30]. Reduced degradation, especially in Δ*pep*A culture filtrates but also in the Δ*pep*B and Δ*pep*E culture filtrates, demonstrated that all three acid proteases are involved in the degradation of PELB in *A. niger*. By recombination, we have also constructed double and triple disruptants. PELB degradation was 0.01–0.002% of that found in the wild-type *A. niger*. At present, the multiple disruptants are being tested *in vivo* for improved heterologous protein production, using several fungal expression–secretion cassettes.

Classical mutagenesis

Considering the number of different proteolytic functions identified and the fact that mutants can be

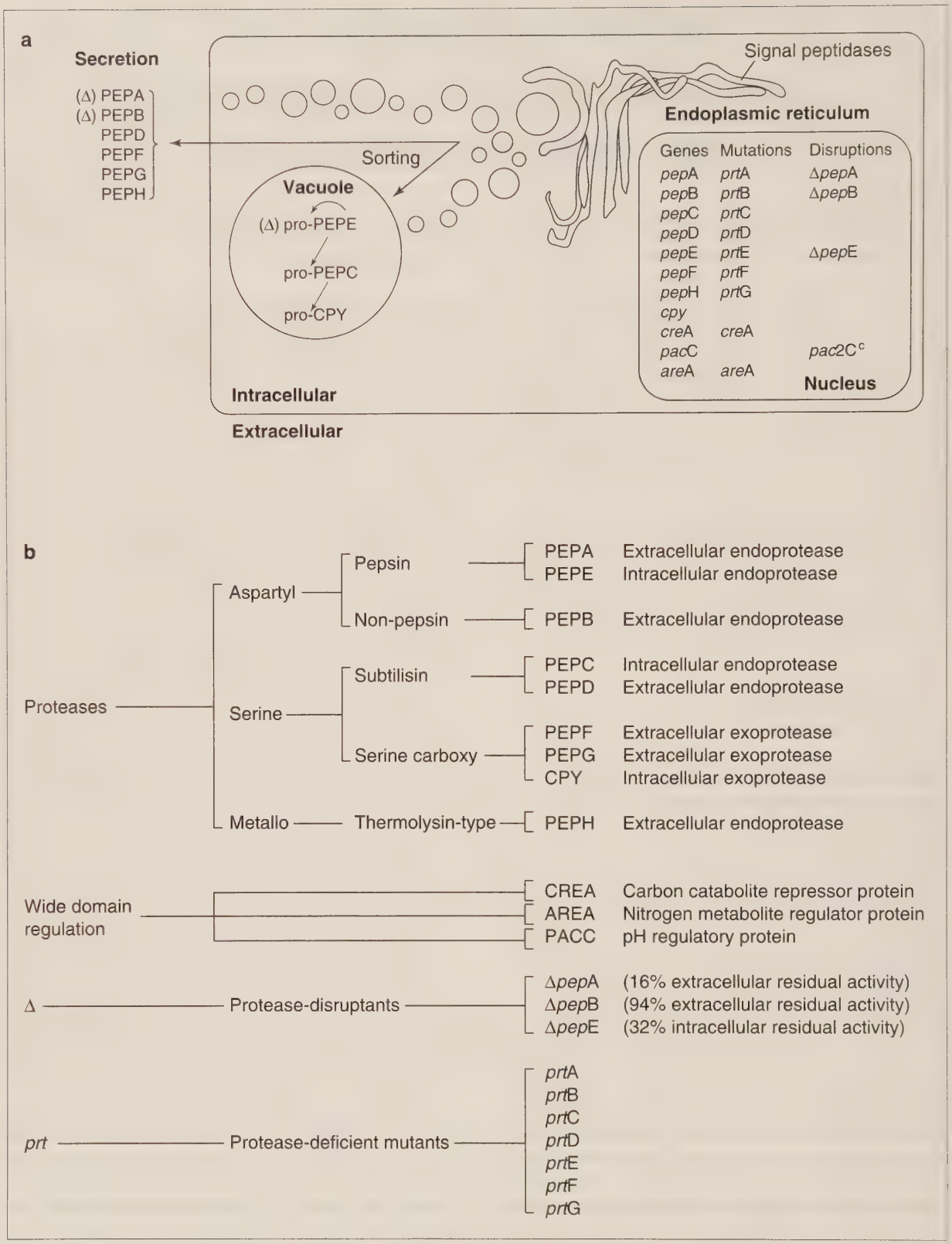

Figure 1

The proteolytic system of *Aspergillus niger*. (**a**) Schematic presentation of the individual functions involved in proteolysis and their localization within the cell. The different genes encoding proteases (*pep*) or regulatory functions (*cre*, *are* and *pac*) as well as the identified protease deficient (*prt*) mutations and the gene disruptions generated (Δ) are listed in the nucleus. The localization of the individual proteases (PEPA–PEPH) is indicated. The cascade-activation mechanism thought to occur in the vacuole is shown. (**b**) Classification of the different functions involved in proteolysis in *A. niger*. The proteolytic enzymes are classified according to their catalytic mechanism. Genetic functions involved in protease expression can be divided into wide-domain regulatory genes and genes that, upon mutation, reduce the expression of proteases (*prt* genes) more specifically. The individual protease gene disruptions and their effects on the proteolytic spectrum are also indicated.

generated easily, we also started a classical mutagenesis program, to generate UV-induced mutants that produce decreased levels of extracellular proteases. Mattern *et al.*[28] have used a similar approach to isolate several protease mutants in *A. niger* that have been used to improve heterologous protein production[11]. Proteases can also reduce the expression yields of certain homologous proteins, and in some cases losses were reduced by the use of specific protease mutants[31,32]. More recently, a number of mutants with decreased extracellular protease activity in *A. nidulans* have been described[34].

Figure 2 summarizes studies on a number of mutant strains selected for reduced extracellular proteolysis on an optimized protein-containing halo-selection medium[30]. After excluding general secretion mutations (which can be identified by screening for the reduction of other extracellular enzymes such as glucoamylase or pectinases), a set of true protease deficient (*prt*) mutants was identified. Genetic characterization resulted in the identification of at least seven independent *prt* functions (complementation groups). Allocation of the individual mutations to four of the eight linkage groups (chromosomes) in *A. niger* showed that one mutation is located on linkage group I (*prt*C), three mutations are located on linkage group III (*prt*B, *prt*E and *prt*G), one mutation is located on linkage group V (*prt*F) and two mutations are located on linkage group VIII (*prt*A and *prt*D). By comparing the allocation results of these *prt* genes with the allocation data for all known protease structural genes and three wide-domain regulatory genes (*cre*A, *are*A and *pac*C), it was shown that most of these *prt* genes cannot be identical to any of these known genes and must encode novel genetic functions involved in protease expression in *A. niger*.

Detailed biochemical analysis of the extracellular proteolytic spectra in the listed *prt* mutants demonstrated residual proteolytic activities varying from 63% (*prt*E15 and *prt*C31) to 2% (*prt*B26). Using protease inhibitors specific for a particular class of proteases, it was demonstrated that for some protease mutants the relative amounts of the different residual proteolytic activities changed (to higher or lower levels) compared with the wild type. These observations clearly demonstrate that specific protease mutations change the overall proteolytic spectrum, and also that compensatory effects of non-mutated proteases emerge. For example, detailed analysis of the residual spectrum of the *prt*F mutants showed increased levels of metalloprotease, which has recently led us to clone a novel protease *pep*H (J. P. T. W. van den Hombergh, PhD thesis). Interestingly, reduced intracellular proteolytic activities in some of the *prt* mutants were also observed.

Using the *in vitro* degradation assay mentioned above we could demonstrate that the PELB stability was improved 500–1000 times in culture supernatants of some single *prt* mutants. This dramatic increase in the *in vitro* stability of the PELB tester protein, resulting from just one single mutation, indicates the potential of these new *prt* mutants to improve protein production *in vivo*. To compare *in vitro* results with *in vivo* expression (and degradation) data, an expression cassette for the *A. niger pel*B gene was used in which the *A. niger pel*B gene was expressed under the control of the *pki*A promoter[34,35]. This enables high constitutive expression of *pel*B on glucose. By introducing this expression cassette into the various *prt* mutants we could demonstrate that in several *prt* mutants the *in vivo* production levels were considerably improved compared with the wild type and, moreover, that the *in vitro* PELB degradation data correlated well with the *in vivo* expression data (J. P. T. W. van den Hombergh, PhD Thesis).

In view of the increased protein production in some *prt* mutants and to assess their future use for the construction of a set of novel production strains, we have characterized these strains at the molecular level to determine which proteases are affected and what the effect is. A comparison of the transcription levels of protease mRNAs for all the cloned *A. niger* protease genes indicates that the protease mutants are a very diverse group of mutations that affect protease expression at different levels (Fig. 2). Whereas some *prt* mutations clearly result in severely reduced, or even a loss of, extracellular protease transcription (e.g. *prt*B26), other *prt* mutations appear to have post-transcriptional effects on the expression of different proteases. For example, for the *prt*A, *prt*D and *prt*F mutations protease gene transcription is not significantly reduced, although there is a strong reduction in extracellular protease activity. In some *prt* complementation groups (*prt*B, D, F and G), mutants were isolated that have less than 15–20% residual extracellular protease activity. This can only be explained if the expression of more than one protease is affected. The molecular expression data therefore suggests that several *prt* genes have either a regulatory or an indirect function (involved in the expression of a number of proteases), or they are involved in a cascade-activation process, in which a single protease is able to activate others. Clearly, the present set of available *prt* mutants exhibits a number of novel functions, which can be exploited further to improve protein production in *Aspergillus*.

Downregulation of protease expression

Considering the variety of proteases and the different processes that are involved in proteolytic steps, it is not surprising that the regulation of proteases is complex. In *A. niger*, the extracellular proteases are subject to complex regulation at the level of transcription. In addition, proteases can be regulated at the translational level or can be synthesized as zymogens to prevent activity before they arrive at the correct location. Furthermore, others are activated via cascade proteolysis and inhibitor proteins may also control their activity. Pathway-specific induction and wide-domain regulation (i.e. regulation of a range of different pathways) provide the means by which a cell normally adapts its overall metabolism to use preferentially those nutrients that can be utilized most efficiently. Especially in the case of many extracellular enzyme systems, specific carbon and nitrogen sources (and/or

Complementation group	–	*prt*A	*prt*B	*prt*C	*prt*D	*prt*E	*prt*F	*prt*G
Linkage group	–	VIII	III	I	VIII	III	V	III
Mutations	–	*prt*A10 A33	*prt*B26	*prt*C7 C14 C31	*prt*D32	*prt*E15 E30	*prt*F28 F29	*prt*G24
Residual protease activities (%)								
Extracellular, pH 4.0	100	21 nd	2	52 32 63	4	63 20	3 3	7
Intracellular, pH 4.0	100	15 nd	11	35 20 12	9	22 36	12 10	20
Intracellular, pH 6.0	100	81 nd	91	92 87 70	54	98 91	157 146	108
mRNA levels								
Mutation	–	*prt*A10	*prt*B26	*prt*C14	*prt*D32	*prt*E30	*prt*F28	*prt*G24
Inducer	B E	B E	B E	B E	B E	B E	B E	B E
*pep*A								
*pep*B								
*pep*C								
Improved *in vitro* PELB stability	1	2–10	500–1000	1–2	500–1000	2–10	500–1000	10–20

Figure 2

Protease-deficient mutants of *Aspergillus niger*. Summary of the results from the genetic, biochemical and molecular characterization of a set of 12 *prt* mutants and their comparison with wild-type N402. Complementation analyses identified at least seven different genetic functions amongst the *prt* mutants, which were each subsequently allocated to one of the eight chromosomes (or linkage groups). Total residual extracellular and intracellular proteolytic activities were determined with cultures in which the (residual) protease spectrum was fully induced to estimate the overall effects of the different *prt* mutations. Analysis of the mRNA levels for the individual proteases in the *prt* mutants was performed on mycelium grown without any repressing carbon and nitrogen sources and in the presence of either elastin or bovine serum albumin to induce protease expression. The combined results from the residual proteolytic activities and protease expression analysis demonstrated that several *prt* complementation groups affected the expression of a number of proteolytic functions, thus implying the involvement of either cascade-activating mechanisms or regulatory effects. Finally, the *in vitro* stability of the PELB protein in the strongest *prt* mutant within a complementation group is indicated in relation to wild type. Abbreviation: nd, not determined.

their degradation products) are able to induce the pathway-specific set of enzymes needed to metabolize a particular substrate.

In the 1970s it was found that depletion of low molecular weight sources of nitrogen, carbon, phosphorus and sulfur results in elevated protease activities in *A. oryzae* and *A. nidulans*[36,37]. Detailed northern expression studies for all cloned proteases in *A. niger* reveal the basis for this phenomenon in more detail. All the extracellular proteases (*pep*A, *pep*B, *pep*D and *pep*F) are only expressed when the preferred carbon and nitrogen sources are not available to the cell[23,36]. In contrast, the cloned intracellular proteases all appear to be constitutively expressed[25]. Interestingly, the extracellular proteases are strictly pH regulated, for example acid proteases are only expressed at an acidic pH.

Recently, the molecular basis for these transcriptional control mechanisms in *A. niger* has been provided by the cloning of three so-called wide-domain regulatory genes, *cre*A, *are*A and *pac*C, which are all closely related to their counterparts found in *A. nidulans*. The *A. niger cre*A gene, encoding a negative regulator protein that mediates carbon catabolite repression[39], was cloned by Drysdale *et al.*[40] The *are*A and *pac*C genes, involved in nitrogen metabolite repression and pH regulation respectively, were cloned in our laboratory[41]. The *are*A gene encodes an activator protein that is needed to alleviate nitrogen metabolite repression (also referred to as ammonium repression). Nitrogen metabolite repression leads to the repression of enzymes and permeases involved in the utilization of nitrogen sources other than the preferred ammonium and L-glutamine[42,43]. The PACC pH regulator protein is only active at an alkaline pH and acts as an activator of alkaline target genes and a repressor of acid target genes, according to the present model[44]. At acidic pH PACC is not active, which leads to derepression of acid expressed genes.

Specific wide-domain regulatory mutations reduce protease expression

We have isolated several *cre*A and *are*A mutants and also constructed a pH regulatory disruptant strain that ensures constitutive expression of alkaline target genes[45]. These mutants provide the tools for a detailed molecular analysis of the effects of wide-domain regulation on extracellular protease expression. Partial escape from carbon catabolite repression and ammonium metabolite repression in such mutants proved the direct involvement of these wide-domain regulatory gene products in extracellular protease expression. Downregulation of acid-protease expression at acid pH in the pH-regulatory disruptant strain demonstrated direct PACC-mediated control. The effects on the transcriptional levels of the major extracellular proteases (*pep*A and *pep*B) in a severe *are*A mutant and in the strain in which pH regulation is modulated are illustrated in Fig. 3. Both mutations result in strongly reduced transcriptional levels of all extracellular proteases, which is reflected by a significant reduction of the total extracellular protease activity.

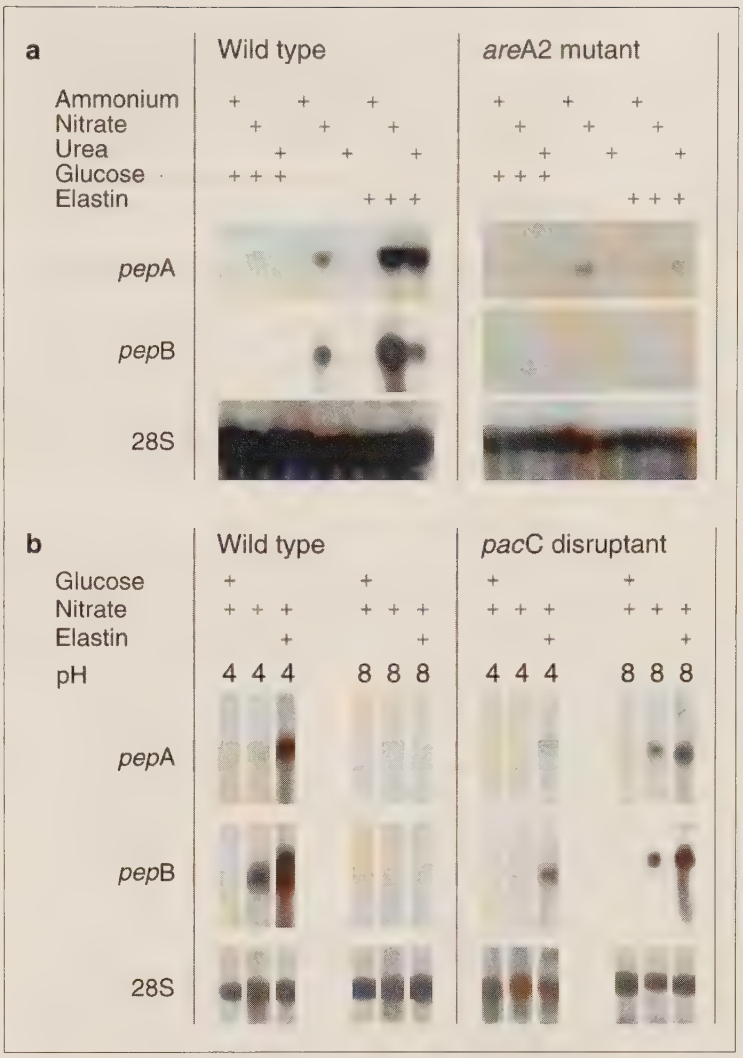

Figure 3

Specific wide-domain regulatory mutations reduce protease expression. The regulation of *pep*A and *pep*B, which encode the major extracellular proteolytic activities, is shown here by northern blot analysis in wild-type and two wide-domain regulatory mutants. Mycelium of the individual strains was transferred into medium supplemented with nitrogen and carbon sources as indicated. (**a**) Effect of nitrogen metabolite repression in *Aspergillus niger* wild type and in an *A. niger are*A mutant. (**b**) Effect of pH regulation in *A. niger* wild type and in an *A. niger pac*C disruptant. Northern blots probed with 28S ribosomal RNA were included to prove loading of identical RNA concentrations.

Optimizing the medium composition

Given the tight control of extracellular protease expression, we set out to devise a strategy to express heterologous proteins under the control of a strong constitutive glycolytic promoter, the pyruvate kinase A (*pki*A) promoter[34]. This promoter allows constitutive expression in media containing high levels of glucose and ammonium, conditions under which most extracellular proteases are repressed[23,36]. Bartling *et al.*[12] recently demonstrated the validity of this concept, using simple expression cassettes based on the *pki*A promoter and terminator that allowed high level expression of a heterologous *Erwinia carotovora* pectate lyase 3, PL3. Inclusion of *A. niger* secretion signals allowed recovery of the functional PL3 from the culture filtrates. Thus this and similar expression cassettes

can be used as *in vivo* tester systems for the expression of heterologous products in *Aspergillus*. Upon relief of carbon catabolite repression and acidification of the culture medium, different proteolytic degradation products of PL3 were observed. This emphasizes the importance of physiological manipulation to diminish the protease problem.

Optimizing expression strategies in protease-deficient strains

Our data clearly show how complex and strictly regulated the proteolytic system in *A. niger* is. We have thus far made some major achievements in reducing the proteolysis problem by systematically integrating three different approaches: (1) cloning and disruption of *pep* genes; (2) classical mutagenesis; and (3) physiological manipulation, as shown in Fig. 4.

The work described illustrates that predictable strain breeding relies strongly on detailed gene expression studies, genetic manipulation and traditional approaches such as mutagenesis and recombination. The development of classical genetics, for instance, has allowed us to construct stable *A. niger* recombinants that contain up to three disrupted protease genes. Multiple *prt* mutants have also been generated by recombination. Double mutants have been obtained that combine a moderate mutation such as *prt*A10 with a second one such as *prt*C14, *prt*E15 or *prt*F28. Even when two relatively weak *prt* mutations were combined, the overall proteolytic activity in these double

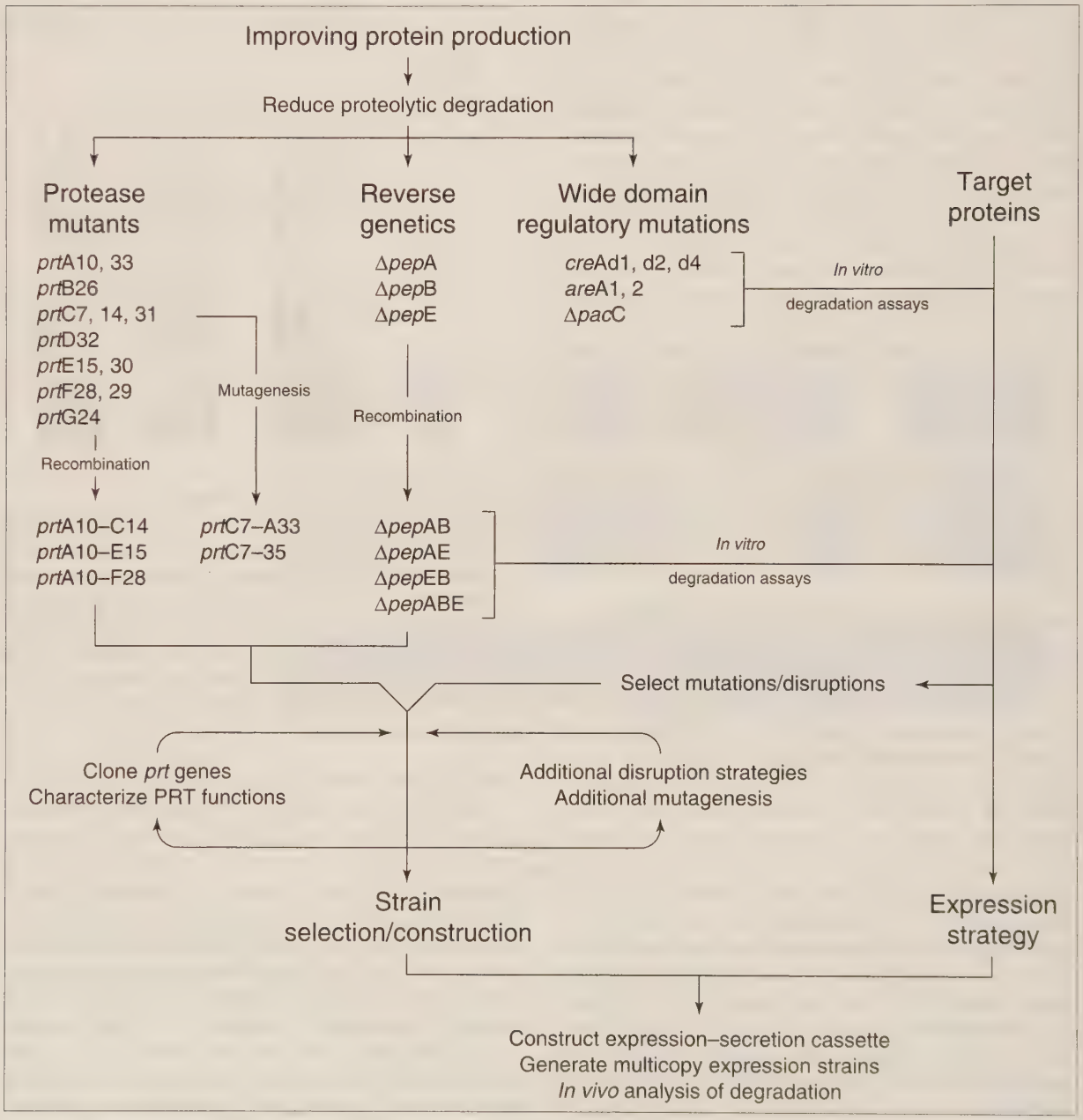

Figure 4

Towards an efficient filamentous fungal expression system. Schematic representation of the integrated approaches to reducing proteolytic degradation in *Aspergillus niger*.

mutants was drastically reduced, illustrating the strength of controlled strain breeding. This integrated approach has resulted in an initial set of multiple-protease-deficient *A. niger* strains, which can be used as a starting point to further optimize expression in this particular host, and the production of proteolytically susceptible proteins from different origins (including bacteria, fungi, plants and vertebrates) is being tested. The *in vitro* and *in vivo* analyses will provide the necessary information regarding which disruptions and/or mutations to combine to improve the production of a particular protein. We plan to make selective combinations of multiple protease disruptions and *prt* mutations to provide a second generation of improved expression strains. We also intend to clone relevant *prt* genes, which will allow detailed molecular analysis of the functions encoded by them and the unravelling of their role, which is still very much *terra incognita*.

Aside from proteolytic degradation, other factors will also be important for further optimization of protein production in filamentous fungi. Although the protein-secretion capacity of *A. niger* has been known for many years, the molecular basis for this observation is still lacking, which led Peberdy[46] to refer to it as 'a highly productive black box'. To manipulate high levels of protein secretion in filamentous fungi will also require more detailed knowledge about the secretion process. Furthermore, the problem of 'product authenticity' will become increasingly important when more therapeutic proteins are expressed. The correct glycosylation of the produced proteins is also a vital issue.

It is obvious that the black box of high-level protein production by filamentous fungi should be opened up further, to develop *Aspergillus* into an even more competitive production system. We expect a major stimulus from the EU Fungal Cell Factory programme, which has recently started to address these questions.

Acknowledgements

We gratefully acknowledge financial support from the EC to J. Visser in the framework of BIOTECH grant BIO 2 CT930174. We thank J. Benen for critically reading the manuscript.

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Serial air-lift bioreactors for the approximation of aerated plug flow

Wilfried A. M. Bakker, Pieter E. M. Overdevest, Hendrik H. Beeftink, Johannes Tramper and Cornelis D. de Gooijer

For bioprocess optimization, plug-flow behaviour is an option in many cases. However, many bioprocesses require oxygen to be supplied into the bioreactor. Plug-flow-like behaviour with aeration is feasible in a series of bioreactors. This article reviews the range of aerated bioreactors available for the approximation of plug-flow behaviour and introduces a new type of serial air-lift bioreactor. The optimal design of cascades of serial bioreactors is also discussed.

Reactor series enable multistep processes in chemical, food and bioprocess engineering to be carried out efficiently. Reactor series may be useful for processes involving two or more independent reactions, each governed by its own kinetics, as in biogas production or nitrification/denitrification[1], as well as those comprising a constant overall reaction stoichiometry that can be described by a single kinetic equation. The latter could be performed in one bioreactor but segregation into two or more bioreactors may lead to a higher product concentration, a larger degree of conversion, a higher volumetric productivity or a combination of these factors[1]. The theoretical explanation for this improvement lies in the fact that with a cascade of a sufficient number of ideal mixers (mixers in which no concentration differences exist) in series, plug-flow behaviour is approximated[2]. With an infinite number of mixers, the approximation would become exact. In a plug-flow reactor (which can be thought of as a long tube, like the gastrointestinal tract) there is no mixing in the axial direction, and both substrate and biocatalyst flow as a plug through the reactor so that the reaction rate in the whole reactor is at its maximum (Fig. 1a). For a continuously operated single vessel (ideal mixer), the rate of reaction is limited by the low concentration of substrate in the reactor, which is equal to that of the outflow[2–4]. For plug-flow reactors, there is a gradient in the concentration of substrate along the reactor; on average, the rate of reaction is thus higher. This means that a continuously operated stirred vessel should be larger than a plug-flow reactor if it is to accomplish the same degree of conversion[2–4], that is, where both reactor types are of the same volume, the substrate is used more efficiently in the plug-flow reactor.

Not all bioprocesses can be carried out in ideal plug-flow reactors, because many of them require oxygen. In general, aeration induces mixing and thus distorts plug-flow behaviour. This drawback can be circumvented by the use of a series of ideally-mixed and aerated bioreactors to approximate plug-flow, but, in terms of capital investment, this may be costly. In this article, we discuss a novel type of serial air-lift loop bioreactors that are incorporated in a single vessel, thus reducing investment costs. Before describing this reactor, the merits and disadvantages of different types of traditional bioreactors will be briefly reviewed.

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Classical bioreactors

A 'conventional' bioreactor is usually a well-stirred vessel or bubble column (Fig. 1b). In the stirred vessel, aeration can be achieved via the head-space, or by additional sparging of air. Mixing and mass transfer are comparable in stirred vessels and bubble columns[3] for most applications. However, the maximum attainable value for mass transfer is higher for stirred vessels, since more power can be introduced by means of stirring. This is especially important for fermentations where the viscosity is above 0.1 N s m^{-2}. However, at larger scales (volumes above 500 m^3) mechanical problems may occur in stirred vessels[3,5].

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PII: S0167-7799(97)01053-6

Air-lift bioreactors form a distinct group of bioreactors that are agitated by air-sparging. In general, the air-lift bioreactor consists of two zones, interconnected at the top and bottom (Fig. 1c). In one of the zones (the riser) air is sparged at the bottom. The air rises and escapes, partially or completely, at the top. Therefore, less air is present in the second zone (the downcomer). The resultant different bulk densities in the riser and the downcomer induce a liquid circulation. Thus aeration and agitation are achieved in this set-up with a low energy input[3,6–8]. Usually, a distinction is made between air-lifts with an internal loop and those with an external loop (Fig. 1c).

Generally, a defined and controlled liquid circulation is obtained in air-lift bioreactors. These conditions comply with the low shear forces necessary for fragile biocatalysts, as in processes involving animal-cell cultures, and so air-lift bioreactors may be useful for the scale-up of such processes[3,6–9]. In terms of mixing and mass transfer, air-lift bioreactors are similar to bubble columns. Thus, air-lift bioreactors are especially attractive for large-scale processes, where a stirred vessel would have mechanical problems because of the required stirrer power[3].

For bioprocesses, the hydrodynamics (i.e. liquid circulation velocity and gas hold-up) in air-lift bioreactors determine important properties like mass transfer (e.g. oxygen supply), heat transfer, shear forces (turbulence) and mixing. These hydrodynamics are greatly influenced by the reactor geometry, and so the dimensions of the different zones of the reactor and their relative sizes are important design parameters. Studies on air-lift-reactor design, application and performance for various biotechnological applications have been recently reviewed[5,6–9], and detailed models are available for rational air-lift bioreactor design[7]. However, most results have been obtained in small vessels (volumes below 0.5 m^3), thus leaving opportunities for confirmative studies on larger-scale applications[7]. With scale-up, the main difference is the wall surface area to volume ratio, which is much smaller than in laboratory equipment. Therefore, the relative influence of the frictional forces on the walls, a key parameter in air-lift design, differs at different scales[7]. This means that liquid velocities will be much higher in taller equipment[7].

Serial bioreactors

Although single-vessel bioreactors are convenient with regard to, for example, measurement, control, sterilization and medium supply, the approximation of plug-flow behaviour achieved by a series of ideal mixers can optimize bioprocesses with respect to objectives such as substrate conversion, product formation or biomass production[1,4,10–17].

Until recently, serial bioreactors consisted of a cascade of separate vessels of classical bioreactors interconnected by hoses and pumps, but a new type of serial bioreactor within one vessel has now been introduced: the Multiple Air-lift Loop reactor[17–19] (MAL: see Box 1 for the merits and disadvantages of this and

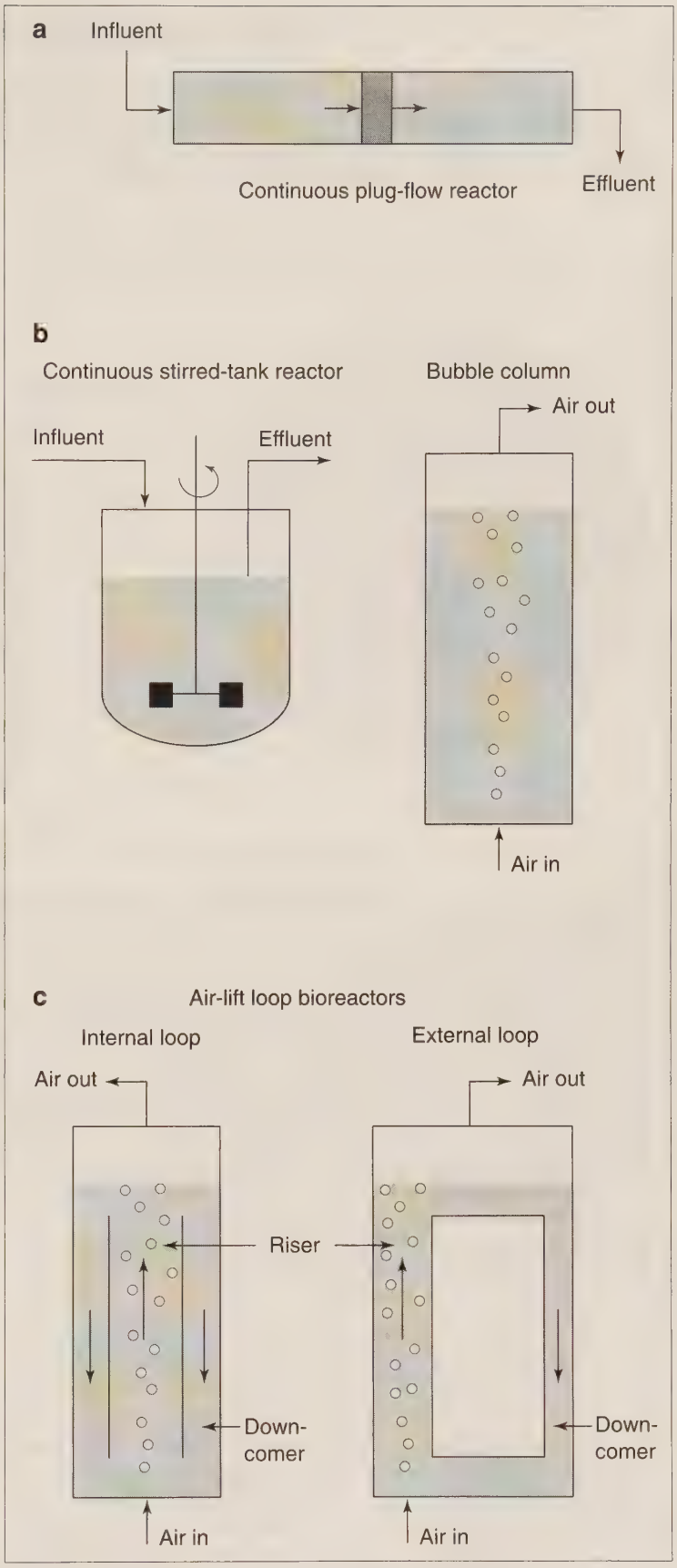


Figure 1
Schemes of classical bioreactors: (**a**) A continuously operated plug-flow bioreactor. There is no mixing: the substrate and catalyst flow like a plug (darker area) through the reactor. (**b**) A continuously operated stirred-tank reactor (ideal mixer) and a bubble column. (**c**) Air-lift loop bioreactors with an internal and an external loop.

Box 1. The multiple air-lift loop bioreactor

The multiple air-lift loop bioreactor (MAL) consists of a series of air-lift loop reactors incorporated into one vessel. The central MAL compartment is a conventional internal-loop air-lift reactor with aeration in the annulus. Subsequent compartments in the MAL are concentric. The annular compartments have a circular baffle that splits them into a riser and a downcomer section. The medium 'travels', driven by gravity, through the cascade via overflows.

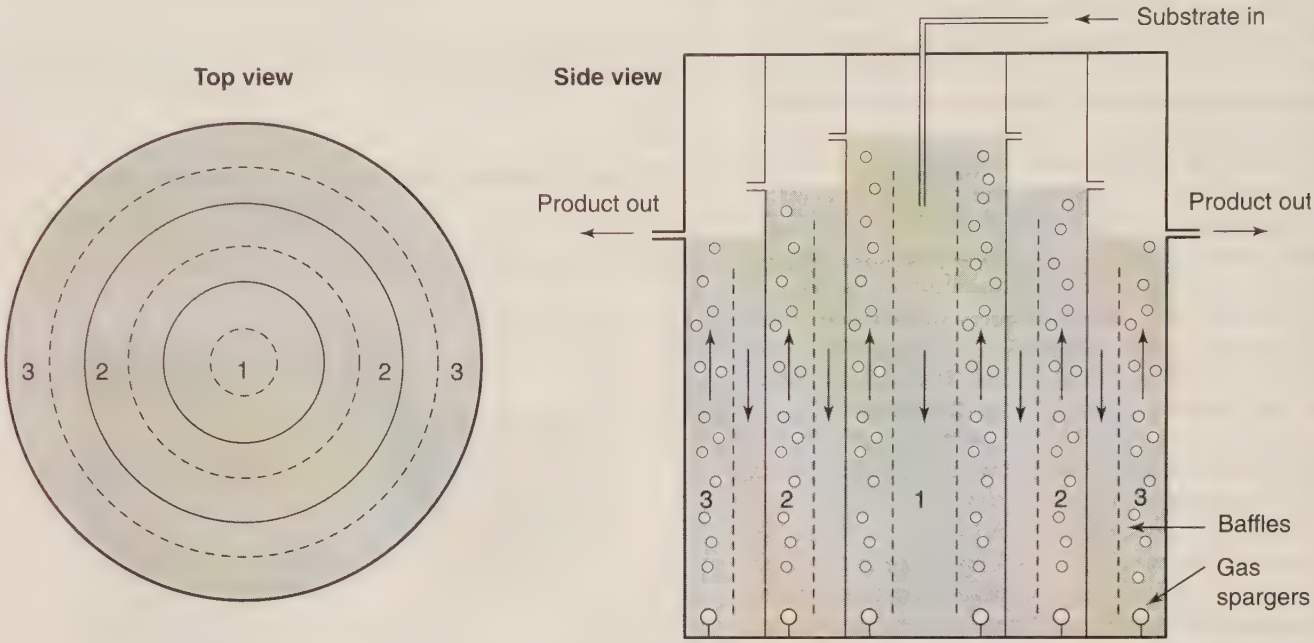


Figure
Three-compartment multiple air-lift loop bioreactor (top and cross-sectional side views).

The advantages of the MAL compared with single air-lift loop reactors in series include:
- many configurations can be constructed for various applications, for example
  (1) for supplying different gases to the subsequent compartments to, for example, carry out aerobic and anaerobic processes in series within one vessel,
  (2) removal of the baffles to obtain a multiple bubble column,
  (3) the medium flow can be directed from the inside to the outside, or vice versa;
- no extra pumps or hoses are needed for medium transport;
- the reactor series can be sterilized as one reactor;
- old reactor vessels can be reused and upgraded to a MAL by introducing walls, baffles and gas distributors;
- inner walls and baffles can be of simple construction because hydrostatic pressure acts on both sides.

The disadvantages for the same comparison include:
- on a lab scale the compartments are narrow, which implies
  (1) difficult cleaning, and
  (2) little space for electrodes and other inserts;
- the gas distributor in the outer compartments is relatively complex.

Nevertheless, there are several cases where the advantages outweigh the disadvantages, as described in this article.

other serial bioreactors). In the Dutch patent application [C. D. de Gooijer (1989) Appln No. 89.01649], describing the MAL in Dutch, many possible configurations for different biotechnological applications were suggested. Initially, attention focused on the possibility of using the MAL to approach the behaviour of an aerated plug-flow reactor. The first design considerations for a MAL were derived from a physical characterization[17,19] and a study with several biological model systems[18,20,21].

Fluid dynamics and design

The new geometry of the MAL was characterized with respect to hydrodynamics (i.e. liquid velocities and gas hold-up), mixing and oxygen transfer. From these studies[17,19], estimations of the hydrodynamic behaviour in a MAL design can be made.

It was found that the outer compartments of the MAL behave like conventional air-lifts with an internal loop. This means that, besides gas hold-up in the riser, there was also a considerable gas hold-up in the downcomer. At all gas flow rates, and in different reactor configurations, the gas hold-up in the downcomer was as high as 88% of that in the riser. This is similar to the ratio found in most studies of conventional air-lift bioreactors with an internal loop [86% (Ref. 17) and 89% (Refs 9,22); but note also the value of 99.7% quoted in Ref. 9]. It should be noted that, at sufficiently

low gas-flow rates, the induced liquid circulation is too low to drag gas into the downcomer[23]. This situation may give the gentle hydrodynamic environment needed for large-scale animal cell culture in air-lift bioreactors[24].

The hydrodynamics of the MAL were described by adapting an existing model for air-lift reactors with an external loop[25] to account for gas hold-up in the downcomer. Good agreement between the experimental measurements and the model estimates was found[17]. Mixing and oxygen transfer in the new reactor configuration were comparable to that in conventional air-lifts with an internal loop. Oxygen transfer was measured by means of both a steady-state and a dynamic method, and both gave comparable results[19].

At high gas flow rates, gas bubbles circulated through the downcomer back to the riser. Interestingly, at low gas flow rates, stagnant gas bubbles were observed in the downcomer. The bubbles may obstruct fluid flow in the downcomer[17,26–29], and thus strongly influence hydrodynamics. Using the general drag–force relationships for bubble, droplet and particle flow developed by Ishii and Zuber[27], a mechanistic explanation for the interfacial drag effects between gas bubbles and the surrounding liquid in the riser and in the downcomer can be given. Based on such considerations, Young *et al.*[29] have estimated the frictional losses in the riser of their air-lift loop reactor, which has an external loop.

Gas hold-up in the downcomer of air-lifts with an internal loop has received little attention until now, and further studies will certainly help to determine the fluid dynamics of air-lift reactors. Recently, Chisti *et al.*[23] addressed this subject. They described the relationship between the gas hold-ups in the riser and downcomer of internal-loop air-lift reactors based on mechanistic principles. However, the frictional-loss coefficient in their model was estimated from a non-mechanistic empirical correlation, which was reported to be uncertain[23] and shown to be invalid for general application[17].

Biological models and cascade optimization

The use of reactor cascades to improve substrate conversion in a single reaction step (compared with the use of a single-vessel of the same overall volume) has been investigated for several biological model systems, including immobilized invertase for sucrose conversion[18] and immobilized growing cells[20] (nitrifying bacteria) for nitrite conversion. In both cases, the reactor cascade gave improved substrate conversion. Sucrose conversion in a three-compartment MAL was 83%, compared with 73% in the single vessel; furthermore, nitrite conversion in a cascade of two air-lift reactors was 86% compared with 61% in the single vessel.

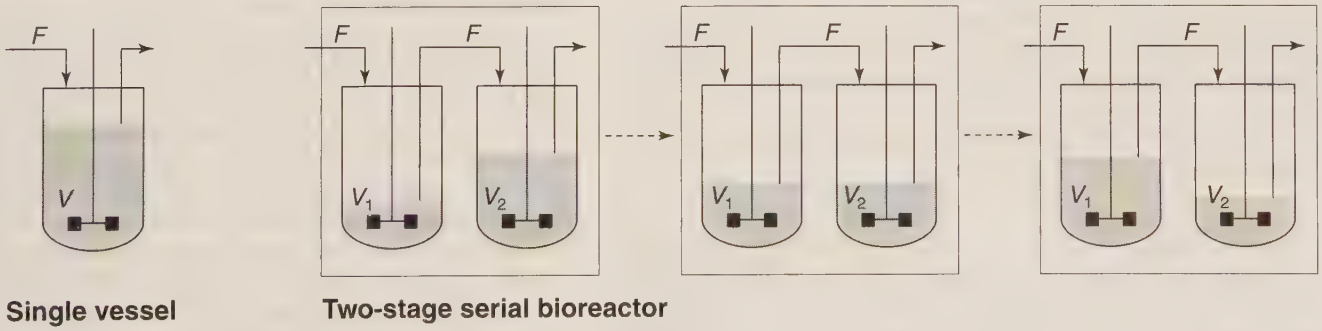
The volumes of the reactors in the series have usually been arbitrarily chosen to be the same, but this is a suboptimal design. Optimal designs have been

Box 2. General optimization scheme for a serial bioreactor

The vessels of a reactor series can be chosen to have different volumes. The best solution of all possible configurations of a reactor series has been calculated according to the chosen optimization criteria. Reactor configurations to be compared are all assumed to be of the same overall volume (i.e. $V = V_1 + V_2$; see Fig.). Then the range of possible feed rates (F) are determined from the kinetic expressions for growth in the same way as described by Pirt[12], which is from zero until washout of the cells occurs. This is equivalent to the whole range of possible overall dilution rates $D_{ov} = 1/\Sigma_{n=1}^{n}(1/D_n)$, with $D_n = F/V_n$.

This range of dilution rates is then divided into a number of steps (about 100–200). At every step in this range of overall dilution rates, the set of balance equations (for cells, substrate and product with the appropriate kinetic expressions) is solved for the unknown substrate concentration by the zero-point finding bisection routine RTBIS of Press *et al.*[31] for every vessel in the series. To find the optimum configuration of a serial reactor, the whole range of possible volume ratios for the reactors in the cascade is then evaluated at this same overall dilution rate (see Fig.), also in small steps. According to the optimization criteria chosen, the optimal configuration is stored, and, subsequently, the next step in the overall dilution rate can be evaluated in the same way. This procedure can be extended to more than two reactors in series.

In all cases: $V_1 + V_2 = V$



Figure

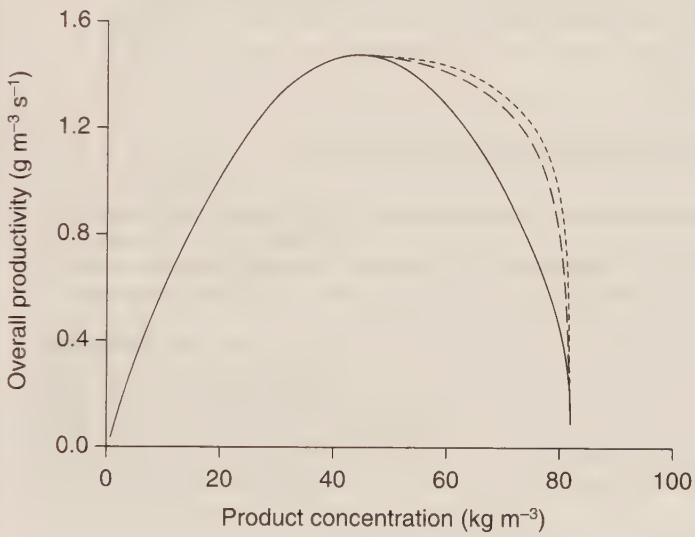
General optimization scheme for serial bioreactors. A single vessel of volume V is compared with a series of two vessels with the same overall volume ($V_1 + V_2 = V$)

Box 3. Bioethanol fermentation

In the example given here, a constant overall reaction stoichiometry is assumed (i.e. substrate conversion is described by a single kinetic equation) and substrate conversion is equivalent to cell growth or product formation. In this case, attention can be focused on product formation instead of substrate conversion.

Shimizu and Matsubara[14] showed that reactor series can be favourable for bioethanol production by yeasts (without taking maintenance into account). Here, the Monod growth kinetics are extended with absolute inhibition by the product ($1-P/P_m$), where P_m is the maximum attainable product concentration.

The overall volumetric productivity [PD = product concentration (P) × overall dilution rate (D)] was calculated at all possible dilution rates until washout occurred. This overall volumetric productivity was plotted as a function of the product concentration (see Fig.). At higher dilution rates, product concentrations were low, while product concentrations became high at the lowest dilution rates. At about 45 kg ethanol m^{-3}, a maximum productivity is seen for a single vessel (Fig.). Shimizu and Matsubara[14] noted that this optimum cannot be improved by using more than one vessel, but they also observed that, at higher product concentrations, a relatively improved volumetric productivity is possible by using more than one reactor. For example, at a product concentration of about 75 kg m^{-3}, productivity is almost 1.5 times higher (increasing from 0.7 to 1.1×10^{-3} kg m^{-3} s^{-1}) when using an optimized series of two reactors instead of a single one. The reactor series was optimized as described in Box 2.



Figure

Volumetric productivity of an ethanol fermentation versus product concentration. With one (——), two (– – –) and three (- - - - -) reactors in series, respectively. Parameter values were as used by Shimizu and Matsubara[14] – K_s = 1.6 kg m^{-3}, $\mu_m = 6.7 \times 10^{-5}$ s^{-1}, Y_{xs} = 0.06, Y_{xp} = 0.16, P_m = 90 kg m^{-3}, and S_0 = 220 kg m^{-3}. Note that at a product concentration of 82.5 kg m^{-3}, all substrate is converted.

Hill and Robinson[15] argue that, with a reactor series, absolute improvement of the volumetric productivity is possible (i.e. above the maximum in the figure). But, the figure shows that, for the kinetic expressions used here, only a relative improvement can be obtained when an additional criterion, like a higher product concentration, is formulated. Therefore, to obtain relative improvements, a combination of at least two optimization criteria has to be chosen[14].

discussed extensively in the literature (for a review see Ref. 1) but only one optimization has been carried out[30], possibly because it requires an accurate, and therefore often complex, kinetic model for which no convenient mathematical expressions can be found. This practical evaluation is certainly a challenge for future research with the MAL, and indeed with reactor cascades in general. A general procedure for choosing an optimal bioreactor-cascade configuration, regardless the complexity of the kinetic model, is outlined in Box 2.

The advantages of a reactor series over a single vessel may be apparent only under certain conditions, for example beyond a given substrate conversion[1]. Furthermore, theoretical differences may be too small to give a significant difference in practice. This was observed for monoclonal antibody production by hybridomas[21], where the accuracy of the measurements was too low to show statistically significant differences between the cascade and a single vessel.

Cells in suspension

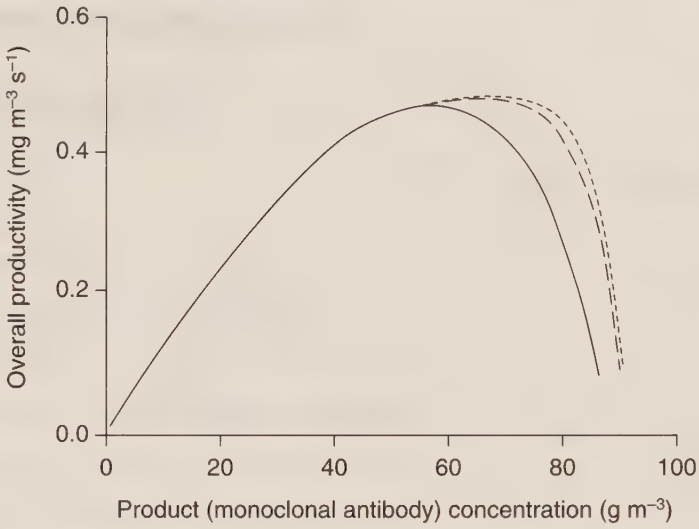
For cells in suspension, such as hybridomas, interesting complications arise when a detailed description of the kinetics is used for bioreactor cascade optimization. Design procedures for cells in suspension with a constant overall reaction stoichiometry (i.e. a single reaction that can be described by a single kinetic equation is involved) have been discussed extensively (e.g. Ref. 1). However, more complex situations, where, for example, the maintenance requirement of the cells, or cell death, has to be accounted for, have been neglected in several theoretical studies[1,4,11,15] for the sake of simplicity. This can lead to suboptimal design of bioreactor series, illustrated first by Shimizu and Matsubara[14]. They found that the maximum product concentration can be improved when the maintenance requirement of the cells is accounted for in optimal serial bioreactor design. They also showed, like Herbert[10], that the maximum attainable overall volumetric product productivity is realizable in a single vessel (Box 3). Furthermore, they found that the relationship between product formation and cell growth can influence this maximum when using an optimally designed series of bioreactors (Box 4). However, no general method for choosing the optimal bioreactor cascade was given.

Interestingly, serial bioreactors can serve as a powerful research tool for kinetic studies of hybridomas[21]. In the second vessel in a series, stable conditions can be obtained that can hardly be reached in a single vessel. This means, for example, that not only growth but also death (apoptosis and necrosis in the case of hybridomas) can be studied under stable conditions in a bioreactor cascade. Furthermore, experiments can be performed at very low growth rates, which eliminates the need for extrapolations to low growth rates when determining kinetic parameters like yield factors and maintenance coefficients[21]. Of course, these findings are also applicable to other cells or microorganisms.

Box 4. Monoclonal antibody production by hybridomas

From continuous cultures, it was seen that viable hybridoma cells not only grow to compensate for dilution, but also for death and lysis[21]. Hence, the net specific growth rate μ_{net} can be described by the rates of growth μ_{true}, death μ_d and lysis μ_l such that $\mu_{net} = \mu_{true} - \mu_d - \mu_l$. Kinetic expressions used in this equation were adaptations of Monod growth kinetics. Further, the maintenance requirements for substrate were not negligible. Thus, a complex kinetic expression described the hybridoma growth, from which the possible advantage of using a reactor cascade for monoclonal antibody (mAb) production can not be seen directly.

Model estimates for a single vessel and an optimized series are given in the Fig., in which the possibility of a relative improvement of the overall mAb productivity at higher mAb concentrations can be observed (e.g. about 1.9 times higher overall productivity at a mAb concentration of 83 g m^{-3}). It should be noted that, in contrast to the bioethanol production example (Box 3), an absolute improvement of the maximum in the overall mAb productivity can be seen when using more than one optimally sized vessel in series. Although only a slight improvement is seen in this example, differences can become more pronounced when other kinetic expressions or other parameter values are used. The improvement seen in the figure is possible because the product formation is not related directly to the substrate converted, but rather to the amount of biomass produced. Therefore, increased biomass concentrations along the reactor series can enhance the overall productivity.



Figure

Model estimates for the overall monoclonal antibody (mAb) productivity by hybridomas as a function of the product concentration for one (——), two (– – –) and three (- - - - -) optimized reactors in series. Parameter values were as used by Bakker *et al.*[21]

Conclusions

This article has indicated several interesting topics for future research on serial air-lift bioreactors that approximate an aerated plug-flow reactor. For the design of air-lift bioreactors with an internal loop, gas hold-up in the downcomer needs to be studied to gain a more detailed description of the fluid dynamics. Practical evaluation of optimal serial-bioractor designs for plug-flow approximations is also required. For cells in suspension, interesting complications arise when a more detailed description of the kinetics is used for bioreactor-cascade optimization. Finally, serial bioreactors can be used as a powerful research tool to develop the kinetic models needed for such optimization studies.

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Probiotic properties of lactic-acid bacteria: plenty of scope for fundamental R & D

Gerald W. Tannock

Probiotic products are marketed widely throughout the world. This is especially true of yogurts that contain strains of lactic-acid bacteria of intestinal origin. Consumption of these products is aimed at promoting the wellbeing of the consumer by impacting on the collection of microorganisms that normally inhabit the intestinal tract. The development of scientifically valid probiotics requires more detailed knowledge of this intestinal microflora than is currently available.

The term 'probiotic' originally referred to a phenomenon observed when two organisms were cultured together, in which substances produced by one organism stimulated the growth of the other organism. These growth-promoting substances were referred to as 'probiotics'[1]. The term was subsequently used to describe living preparations of microbial cells that could be administered to animals, including humans, with the aim of promoting the health of the consumer[2]. This latter concept is derived from the rather philosophical observations made by Elie Metchnikoff and others earlier this century (Box 1). In the case of farm animals, faster weight gain for the same amount of food consumed (growth promotion, feed efficiency) have been of primary importance.

By definition, a probiotic is a 'live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance'[2]. The marketing of probiotics for human consumption relies heavily on this definition. Specific health-related claims are not usually made in relation to the products, but general statements such as 'helps maintain a healthy balance of beneficial bacteria', 'stabilizes the intestinal microflora and modulates its function', 'promote the positive balance of the intestinal flora' are included on labels and in advertising brochures. Implicit in the definition and in the marketing of probiotics is that the ingested microorganisms impact on the composition of the normal microflora of the intestinal tract. The manufacture and marketing of probiotic yogurts (Acidophilus–Bifidus yogurts) has increased dramatically worldwide in recent years, and a desire to ascribe some scientific validity to these products, rather than to rely on a theoretical concept, is apparent in the dairy-food industry. Establishing the scientific validity of probiotics will require a substantial commitment from industry to supporting fundamental studies of microorganism–host relationships. The results of these fundamental studies will define the health-related aspects in relation to lactic-acid bacteria that should be studied in the future.

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The intestinal microflora

The intestinal tracts of all mammalian species that have been studied, appropriately harbour a complex collection of microorganisms, mostly anaerobic bacterial species, that is known as the intestinal microflora[5]. This microflora is mostly located in the large bowel in monogastric animals such as humans (Box 2). The predominant bacterial members of the microflora attain population levels of about 10^{10} per gram (wet weight) of contents, so that the human colon, for example, contains at least 10^{12} living bacterial cells. About 400 bacterial species have been detected in human faeces (the faecal microflora is representative of the microflora of the colon[6]) but 30 to 40 species constitute 99% of the collection in any one human subject[7]. While the numerically predominant genera of bacteria detected in the faeces of different individuals are the same, there is variation in the occurrence and population size of bacterial species[6]. The complex composition of the intestinal microflora is even more apparent from the results of recent studies in which populations of bacteria were analysed at the level of bacterial strains. Species can be divided into numerous strains on the basis of differences in the occurrence of short sequences of genomic DNA at which enzymes (restriction endonucleases) will cut the polynucleotide strands. Variation in the number and location of these sequences in different strains provides

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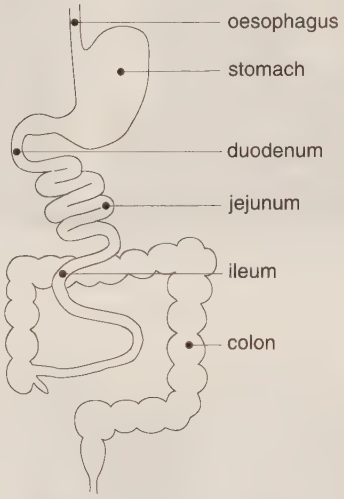
PII: S0167-7799(97)01056-1

Box 1. Elie Metchnikoff (1845–1916) and the elixir of life

Elie Metchnikoff, Nobel Laureate (for his discovery of phagocytosis) of the Institut Pasteur, Paris, was interested in the scientific basis of ageing. Couched in the natural history perception of science prevalent in his time, Metchnikoff's books convey an overall message that the intestinal microflora could be detrimental to the host. According to Metchnikoff, the large bowel harboured microorganisms that produced substances that were toxic to the vascular and nervous systems. The toxic substances, as a result of absorption into the bloodstream, contributed to the ageing process. Thus, intestinal microorganisms were the aetiological agents of 'autointoxication' because they produced ammonia, amines and indole as a result of protein hydrolysis (putrefaction) in the digestive tract. Metchnikoff's remedy for autointoxication was radical: he advocated surgical removal of the large bowel. However, a more acceptable remedy was to modify the intestinal microflora by replacing or diminishing the number of putrefactive microorganisms in the intestine. This could be accomplished, it was suggested, by enriching the microflora with bacterial populations that obtained energy by the fermentation of carbohydrates rather than hydrolysis of proteins. Lactic-acid-producing bacteria were favoured as fermentative microorganisms for this purpose because it had been observed that the natural fermentation of milk by these microorganisms prevented the growth of non-acid-tolerant microorganisms, including those with proteolytic activity. If a lactic fermentation prevented the putrefaction of milk, would it not have the same effect in the digestive tract if appropriate microorganisms were used? The inhabitants of Eastern European countries, some of whom were, apparently, extremely long-lived, consumed fermented milk as a constant part of their diet. Thus, yogurts were introduced to Western Europe as health-related foods[3,4].

Box 2. Bacterial genera that are commonly detected as components of the intestinal microflora of humans



Bacteroides
Gram-negative, non-spore-forming bacilli. Obligate anaerobes. Metabolic products include combinations of acetic, succinic, lactic, formic or propionic acids. If *N*-butyric acid is produced, isobutyric and isovaleric acids are also present.

Bifidobacterium
Gram-positive, non-spore-forming, nonmotile bacilli, sometimes with club-shaped or spatulated extremities. Obligate anaerobes. Acetic and lactic acids are produced primarily, in the molar ratio 3:2. Glucose is degraded exclusively and characteristically by the fructose-6-phosphate 'shunt' metabolic pathway.

Clostridium
Gram-positive bacilli that form endospores. Obligate anaerobes.

Enterococcus
Gram-positive cocci. Facultative anaerobes. Lancefield group D. Can grow in 6.5% NaCl broth and in normal broth at pH 9.6.

Eubacterium
Gram-positive bacilli, non-spore-forming. Obligate anaerobes. Produce mixtures of organic acids including butyric, acetic and formic acids.

Fusobacterium
Gram-negative, non-spore-forming bacilli. Obligate anaerobes. *N*-butyric acid is produced but isobutyric and isovaleric acids are not.

Peptostreptococcus
Gram-positive cocci. Obligate anaerobes. Can metabolize peptone and amino acids.

Ruminococcus
Gram-positive cocci. Obligate anaerobes. Amino acids and peptides are not fermented. Fermentation of carbohydrates produces acetic, succinic and lactic acids, ethanol, carbon dioxide and hydrogen.

Lactobacillus
Gram-positive bacilli, non-spore-forming. Grow best under anaerobic conditions. Lactic acid is a major product of glucose fermentation.

Escherichia coli
Gram-negative rods, facultatively anaerobic. Citrate not utilized. Carbohydrates fermented to lactic, acetic and formic acids. Part of formic acid is split by a complex hydrogenase system to give equal amounts of carbon dioxide and hydrogen. Lactose is fermented by most strains but fermentation can be delayed or absent. Motile by means of peritrichous flagella or nonmotile.

Box 3. You can be identified on the basis of your intestinal microflora

In recent studies carried out in the author's laboratory, the compositions of the bifidobacterial and lactobacillar populations inhabiting the intestine of ten healthy humans were analysed by differentiating between bacterial strains according to their genetic fingerprints. Faecal homogenates were prepared, diluted serially and selective agar plate cultures set up. After incubation and enumeration of the cultured bacteria, randomly selected colonies were subcultured and fingerprinted. The proportion that each strain contributed to the total bifidobacterial or lactobacillar population was thus determined. Faecal samples were collected from two of the subjects at monthly intervals over a 12-month period. These studies revealed that each human had their own unique collection of *Bifidobacterium* and *Lactobacillus* strains. In some subjects, a relatively simple collection of strains was present; in others it was complex. Subject 1, for example, harboured a simple and constant collection of bifidobacterial strains (Fig. 1) whereas Subject 2 had a complex bifidobacterial microflora that appeared to fluctuate in composition (Fig. 2)[8]. (Figures reproduced courtesy of the American Society for Microbiology.)

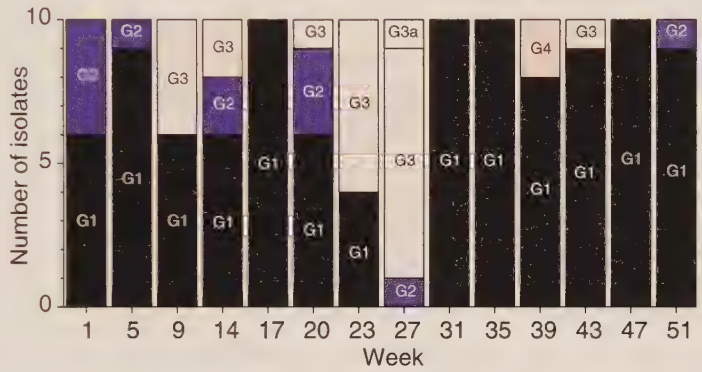


Figure 1

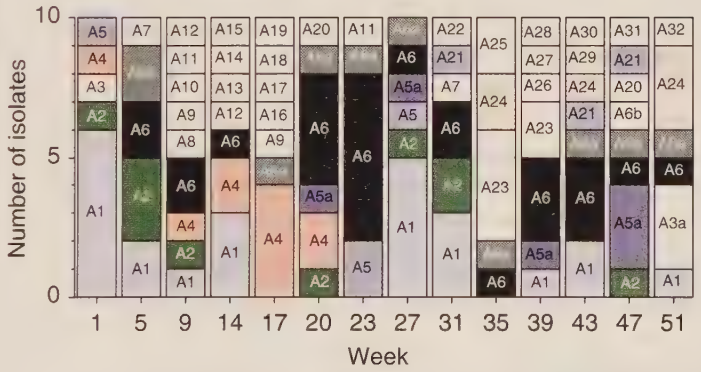


Figure 2

a method of genetic fingerprinting: DNA from a particular bacterial isolate, when digested with an appropriate restriction endonuclease, gives a characteristic pattern of different-sized DNA fragments after agarose-gel electrophoresis. Molecular analysis of two lactic-acid-producing genera that are members of the intestinal microflora has shown that each human harbours their own unique collections of bacterial strains (Box 3)[8]. This technology, combined with the use of polymerase chain reaction methods[9] and oligonucleotide probes[10], now provides the means by which the detailed analysis of the effect of consuming a probiotic product on the compostion of bacterial populations already resident in the intestinal tract can be made.

Choice of strains for use as probiotics

Lactic-acid-producing bacteria are common components of probiotics (Table 1). They are popular choices because of the historical belief that these bacteria are desirable members of the intestinal microflora, arising from the fact that lactic-acid bacteria have long been used in the manufacture of dairy foods and are thus 'generally regarded as safe', and because the consequent large-scale-culture and preservation methods for lactic-acid bacteria in a viable state have already been developed by the dairy industry. The choice of strains to be included in probiotic products has largely been decided on the basis of whether they are amenable to industrial handling and if they will remain viable for a suitable time in the prepared product. While these are legitimate reasons for choosing a strain for industrial use, information as to the ability of the bacterial strain to fulfil the definition of a probiotic (i.e. whether it modifies the intestinal microflora) is, strangely, lacking. Studies demonstrating the recovery of an administered probiotic strain from faeces have been published, but detailed analyses of the microflora following consumption of a commercial product are generally unavailable. Indeed, the detection of probiotic strains may have been somewhat unreliable in studies to date because it has been based on bacterial-colony morphology[11]. However, experimental animal studies conducted in recent years have provided biochemical markers that could be used to show whether an administered probiotic has influenced the intestinal ecosystem. These studies utilized mice that do not harbour lactobacilli as part of their intestinal microflora[12]. Comparisons of the characteristics of *Lactobacillus*-free mice with counterparts that had been intentionally colonized with *Lactobacillus* strains, but in which an otherwise-identical microflora was present, have demonstrated that lactobacilli have major influences on intestinal biochemistry. The biochemical markers identified by these comparisons are bile-salt-hydrolase activity and the concentration of unconjugated bile acids in intestinal contents (increased in the presence of lactobacilli)[13,14], and azoreductase and β-glucuronidase activity (reduced in the presence of lactobacilli)[15,16].

Several *Lactobacillus* species have been included in probiotic products (Table 1). Theoretically, the choice of species should be dictated by the animal species for which the product is intended, because the composition of their microfloras may differ. In practice, comprehensive taxonomic studies based on the molecular phylogeny of lactic-acid components of the intestinal microflora of different animal species have not been made. *Lactobacillus acidophilus*, until recently a catch-all species composed of disparate isolates and used widely to describe the *Lactobacillus* components of probiotics,

has been split into six new groups (*L. acidophilus*, *L. amylovorus*, *L. crispatus*, *L. gallinarum*, *L. gasseri* and *L. johnsonii*)[17]. The exact identity of the lactobacilli included in probiotics is often not known. *Lactobacillus reuteri* is considered by some researchers to be the most prevalent heterofermentative *Lactobacillus* species inhabiting the intestinal tract of humans and other animals[18]. Clearly, extensive research needs to be conducted in this area.

One of the desirable properties sometimes listed for probiotic strains is that they should be resistant to antibiotics[19]. The rationales for this approach are that probiotic products could be used to reconstitute the intestinal microflora of patients suffering from antibiotic-associated colitis or be fed to farm animals administered 'growth-promoting' concentrations of antibiotics in their food. Because a residue of antibiotic could be present in the intestine of the patients or of the farm animals, only antibiotic-resistant probiotic strains would be able to colonize the ecosystem. This approach would surely compound the existing problem of antibiotic resistance in bacteria of medical importance. Although the probiotic bacteria would be unlikely to cause a pathological process, they could serve as a reservoir of antibiotic-resistance determinants that could be transmitted to pathogens. Transmission of antibiotic-resistance determinants on plasmids has been demonstrated to occur from lactobacilli to other Gram-positive bacteria under laboratory conditions and in the intestinal tract[20,21].

Which are best: lactobacilli or bifidobacteria?

Lactobacilli have the longest history of use as probiotics and are still the most common ingredients of those intended for consumption by farm animals, notably pigs and poultry. This choice of probiotic bacteria seems appropriate, because the digestive tract microfloras of these animal species are particularly rich in lactobacilli when the pigs and poultry are maintained under optimal conditions of animal husbandry[22]. *Bifidobacterium* species are now nearly as common as lactobacilli in yogurts, presumably as a result of the realization that the human intestinal tract harbours larger populations of bifidobacteria than lactobacilli. What functions do bifidobacteria perform in the intestinal ecosystem, how do these activities influence the consumer and, therefore, what is the scientific rationale for including them in probiotics? These are, currently, unanswered questions.

The presence of both lactobacilli and bifidobacteria as members of the intestinal tract microflora of the majority of humans logically requires that the ingested probiotic strain have some health-promoting characteristic that the resident lactic-acid bacteria lack. The probiotic strain would need to be able to compete sufficiently with the resident lactobacilli or bifidobacteria for the expression of the beneficial attribute. It is therefore necessary to determine the proportion of the lactobacillar or bifidobacterial population that the probiotic strain achieves in the intestinal tract in order for the product to have scientific validity.

Table 1. Examples of microorganisms used in probiotic products

Products for humans	Products for farm animals
Lactobacillus acidophilus	*L. acidophilus*
Lactobacillus casei Shirota strain	*L. casei*
Lactobacillus delbrueckii subspecies *bulgaricus*	*L. delbrueckii* subspecies *bulgaricus*
Lactobacillus johnsonii	*L. plantarum*
Lactobacillus reuteri	*L. reuteri*
Lactobacillus rhamnosus	*Bifidobacterium bifidum*
Bifidobacterium adolescentis	*Bacillus subtilis*
Bifidobacterium bifidum	*Streptococcus thermophilus*
Bifidobacterium breve	*Pediococcus pentosaceus*
Bifidobacterium longum	*Enterococcus faecium*
Bifidobacterium infantis	*Saccharomyces cerevisiae*
Streptococcus thermophilus	*Aspergillus oryzae*
Saccharomyces boulardii	*Torulopsis* spp.

Probiotics and the immune system

While the purported benefits of the consumption of probiotic products are several, and often astounding[23], the relationship between microorganisms inhabiting the intestinal tract and the immune system of their host can be investigated scientifically. Interest in the ability of lactic-acid-producing bacteria to stimulate the defence mechanisms of the body originated in the use of extracts of fermented mistletoe to treat cancer patients in the 1920s. The extracts were demonstrated to stimulate the immune system of experimental animals; the stimulating components were the cells of lactobacilli[24]. This immunological effect, which principally involves the activation of macrophages, is due to constituents of the bacterial cell wall[25]. Lactobacilli are Gram-positive bacteria and thus their cell wall is composed mostly of peptidoglycan. Degradation products of peptidoglycan include muramyl peptides, which can be detected in the systemic tissues[26] and have pharmacological activity affecting sleep patterns, body temperature and appetite[27–29]. Additionally, muramyl peptides are strong adjuvants, as can be seen from the fact that they enhance immunological reactivity[30].

Lactobacilli, and other members of the intestinal microflora present in the digestive tract contents, are separated from the tissues and organs of the host by the intestinal epithelium. The epithelium is composed of a layer, only one cell thick, of column-shaped cells referred to as enterocytes. How do *Lactobacillus* cell wall substances and other constituents gain access to the immune system of the host? Perhaps the most probable explanation is the passage of particulate or soluble *Lactobacillus* substances into the accumulations of immunological tissue, the Peyer's patches that are present at intervals along the intestinal wall[31]. The patches are separated from the intestinal lumen by M cells that appear to have an antigen-sampling role. Whole bacterial cells may also pass (translocate) from the intestinal lumen into the blood or lymphatic systems via the Peyer's patches or temporary breaks in the epithelial barrier. Bacterial translocation is rare in

healthy, adult animals but may be more common in young animals[32]. The lactobacilli and other microorganisms may be able to stimulate the immune system without leaving their lumenal habitat since enterocytes, it is now recognized, are immunocompetent cells and produce chemical messengers (cytokines) involved in the regulation of the immune system. When exposed to certain bacterial cells or their products, enterocytes have been shown to increase the expression of genes encoding interleukin 8, tumour necrosis factor α, monocyte chemotactic protein 1 and granulocyte–macrophage colony-stimulating factor[33]. These substances have well-established roles in the attraction and activation of polymorphonuclear leukocytes and macrophages. Investigation of the effect of *Lactobacillus* strains on the expression of cytokines by enterocytes should therefore be an area of high priority for probiotic research.

Conclusion and prospects

The derivation of efficaceous probiotics requires substantial research and development, especially at the level of fundamental science. An investment by biotechnological companies in obtaining knowledge of the microbial ecology of the intestinal tract will pay dividends for them, because the results of this research will provide a basis for firm claims of efficacy to be made in the marketing of probiotic products. The incorporation of intestinal strains of lactic-acid bacteria into yogurts provides the opportunity to market a 'functional food': one that combines nutrition with another beneficial consequence for the consumer.

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Focus on animal cells

Animal Cell Technology. From Vaccines to Genetic Medicine

edited by Manuel J. T. Carrondo, Bryan Griffiths and José L. P. Moreira, Kluwer Academic Publishers, 1997. UK£246.00 hbk (xviii + 811 pages) ISBN 0 7923 4321 2

Multidisciplinarity became a mantra that was frequently chanted by the funding agencies. Few fields of research are as multidisciplinary as animal cell technology – chemical engineers tinkering with the design of fermentation tanks to molecular biologists redesigning the glycosylation machinery of the endoplasmic reticulum, it's all here. This book, which records the Proceedings of the 14th Meeting of the European Society for Animal Cell Technology, starts with transcripts of talks given by the keynote lecturers at the meeting. In 1996 the lecturers were C. Weissmann (Zurich), who talked about the molecular biology of prion diseases, W-D. Schleuning (Berlin), who discussed applications of animal cell technology to drug discovery and pharmacology, and H. F. Lodish (Cambridge, USA), who talked about the biogenesis of secretory proteins and cell-surface receptors in mammalian cells (unfortunately, C. Weissmann only provided an abstract of what must have been a fascinating talk). The book is then grouped into sections, corresponding to the sessions of the meeting. The titles of the sessions included: 'Cells and vectors for genetic medicine'; 'Animal cell vaccines – present and future'; 'Animal cell process engineering'; 'Recombinant proteins: biosynthesis and processing'; 'Tissue engineering and biomedical devices'; and 'Cell physiology and engineering'. Each section contains mini-papers in a fairly standard format of abstract, introduction, materials and methods, etc. For those papers selected for oral presentation, there is also, at the end of the paper, a transcript of the discussion that followed the presentation. Although there is no colour, the quality of the photographs and figures is good. My only criticism is of the subject index, which is fairly sparse.

In the words of the editors, animal cell technology has become a 'central resource for innovation in pharmaceutical research and development'. This book illustrates admirably why this is so. With over 120 contributed papers, it provides a comprehensive snapshot of the field in May 1996 and thus should be a useful reference work for those engaged in research in this area.

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Solutions to a sticky problem

Immobilization of Enzymes and Cells

edited by Gordon F. Bickerstaff, Humana Press, 1996. US$74.50 hbk (384 pages) ISBN 0 89603 386 4

This new book ends a silence of more than ten years in the scientific literature of this field. The last similar book was edited by J. Woodward in 1985 (also entitled *Immobilization of Enzymes and Cells*) and appeared in the *A Practical Approach* series. To my knowledge, this was the last collection of true examples of enzyme- and cell-immobilization techniques. Another book that approached the topic, in a way, was *Biosensors*, edited by A. Cass in 1990 and in the same *A Practical Approach* series, keeping the practical spirit as its guiding line. However, in a world of exploding technology and rapid progress, the publication of such a book should be reconsidered every few years.

Obviously, the aim of this book was not an exhaustive theoretical coverage of the immobilization methods available, but rather a wide review of checked and optimized procedures. The innumerable novel substrates, materials, substances and techniques overwhelm researchers when faced with the difficulty of making a choice. Being an electrical engineer myself, I have often felt lost in this jungle, and I began to appreciate the procedures that were reproducible and easy to control, rather than those with spectacular but more random yields. Consequently, an immobilization process described in detail, so that it can be reproduced by everyone, seems to me far more valuable than a chain of exotic reactions outlining a highly efficient immobilization principle. Moreover, most of the time, the key to success lies in small tips, the trade secrets accumulated over years of trial and error. Therefore the *Notes* ending each chapter of this book are of particular interest. An attentive reader may find in these *Notes* the answers to many of the questions that arise during the reading of the procedures. Last but not least, most of the immobilization techniques described are accompanied by appropriate evaluation methods. This is a section that is generally lacking in the theoretical books on immobilization and yet it is the only way to isolate the problems correctly.

A variety of different substrates (from glass, nylon and sephadex to thermoresponsive polymers, magnetic beads and red blood cells), matrices (from bovine serum albumin and agarose to hydrogels

and polyelectrolyte complexes) and reactions (from silanization and succinilation to chelation of metal ions) are described. Assay methods are given in several places. The problems of steric hindrance are also treated, as are ideas on enzyme stabilization. In fact, there is a plethora of choices so one can hardly complain of not finding the right issue but, still, nothing is perfect.

I would have liked to see more formulae, but perhaps this is my engineering background. However, I appreciated the clarity of the chapters with a short theoretical description. I would also have liked to have seen more references to the source of the information: the work of Weetall or Arkles was never mentioned in the silanization procedures, although there are exhaustive references on this topic from the late 1970s. Moreover, only aqueous silanization is mentioned and, of these procedures, only the γ-aminopropyltriethoxysilane, although recently many silanes with the most exotic functional moieties have been produced: thiol, glycidoxy, succinimidil, cyano, thiocyano and so on. I would like to see practical considerations of such issues.

There are a lot of problems in the almost universally used glutaraldehyde crosslinking. Some of these problems originate from Schiff-base formation, yet the Schiff-base-reduction problem is mentioned only in Chapter 5, which I found particularly interesting for its Notes. It is also the first to mention alternative silanization methods, the problems due to the hydrolysis of the reactive groups, and yields. However, the visual inspection of monolayers on surfaces (widely used on smooth surfaces, such as silicon dioxide grown in planar microelectronic technology) is rather complicated and hence the fifth chapter really only applies to porous, rough surfaces.

As a general observation I would have liked to see some comparisons between the results of different methods, reasons for choosing given methods and hints on when certain methods are better than others. Nevertheless, I see this book as a valuable tool for researchers in the many areas requiring enzyme and cell immobilizations. As the advantages of immobilized cells and enzymes were recognized, intense activity in various areas (biotechnology, biology, food processing, analysis, medicine) became evident. Every researcher faces a novel, complex problem and 'rediscovering America' is still time-consuming and frustrating. This is the value of this book, which should be frequently reconsidered and updated.

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Useful recipes for antibody engineers

Antibody Engineering: A Practical Approach

edited by J. McCafferty, H. R. Hoogenboom and D. J. Chiswell, IRL Press, 1996. (325 pages) Pbk: UK£27.50 ISBN 0 1996 3592 7. Hbk: UK£55.00 ISBN 0 1996 3593 5

A number of books on this topic have recently appeared, but *Antibody Engineering: A Practical Approach* offers complete and up-to-date coverage of most of the techniques used to engineer antibodies. The book starts with two chapters on the generation of antibody-V-gene repertoires and bacteriophage display, including mutagenesis strategies and selection protocols. These chapters contain a rigorous description of these rapidly evolving technologies. In the following chapter, the use of yeast artificial chromosomes (YACs) for the generation of transgenic mice expressing human antibody genes is described. Again, this is an exciting new technology that has vast potential for the production of human antibodies in mice, with the mouse, rather than man, carrying out the affinity maturation.

Different approaches to the measurement of antibody affinities are discussed in the next two chapters. Firstly, ELISA-based methods are described, followed by the use of surface plasmon resonance and Pharmacia's BIAcore (although the discussion is restricted to the generation of BIAcore data, it is also applicable to the use of biosensors that rely on resonant mirrors for detection, such as the IASys system). These methods are clearly presented, and the limitations (primarily due to mass-transport effects) of the BIAcore are touched upon. It is useful to have both methods within the same text, as a complementary approach to the use of the BIAcore or similar real-time biosensor is valuable both to confirm surface plasmon resonance data and for those who do not have access to biosensors of any kind.

A comprehensive chapter on the analysis of human-V-gene sequences is followed by chapters on humanization (complementarity-determining-region, or CDR, grafting) and the newer approach of generating human antibodies by guided selection using bacteriophage display. This latter approach is based on chain shuffling and offers advantages over CDR grafting, such as the generation of a variety of antibodies with different affinities and fine specificities.

The focus then changes to the constant region and the manipulation of effector functions. One chapter covers antibody-dependent cell-mediated cytotoxicity (ADCC) and complement fixation; the structural requirements for these activities, and how to measure them, are described. At this point in the book, it might also have been

useful to have a section/chapter on the pharmacokinetic analysis of antibodies, as it is clear that this is an important parameter for their use in therapy.

The final chapters deal with the expression of antibodies in *Escherichia coli* and mammalian cells. For *E. coli* expression, both export into the periplasm and refolding from inclusion bodies are described. In particular, Chapter 10 offers a detailed discussion of the factors that govern expression levels and describes many useful tips for those interested in expressing recombinant antibodies on either a small or a large scale.

In summary, this book has detailed protocols in the usual format of the *Practical Approach* series and offers a comprehensive description of the current technologies that are being used. It is a valuable addition to the bookshelf of anyone who is interested in the practicalities of engineering antibodies.

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Bioinformatics online

Methods in Enzymology, Vol 266: Computer Methods for Macromolecular Sequence Analysis

edited by R. F. Doolittle, Academic Press, 1996. US$110.00 (711 pages)
ISBN 0 12 182167 6

The relationship between modern molecular biology and informatics is unique – there is no other branch of science in which the competitive nature of research requires that bench scientists should have immediate access to 10^{10} bits of specific information. Developments within biology, such as the publication of complete genome sequences, generate rapid change in research methods, and there is a close coupling with informatics developments that also generate a high rate of change, such as the growth of Internet resources and the never-ending improvement in the cost:performance ratio of microprocessors.

All of this must make it an almost impossible task to edit a volume of *Methods in Enzymology* devoted to computerized methods for macromolecular sequence analysis that will fully meet the aspirations of the series – to be thorough, timely, authoritative and of lasting value. The problem is aggravated by the egocentricity of some workers in this field, who might say, like the writer caricatured in *Punch*, 'I don't read papers, I write them'. Russell F. Doolittle first took on this task with considerable success with Volume 183, published in 1990; according to the Preface of the volume under review (266), enough of this material is now outmoded that an updated volume covering the same ground is required.

Considered on its own, the new volume contains a great deal of useful material, of several types. There are articles bringing together reference material about a particular method that is otherwise scattered in a number of, often less accessible, publications. Examples are the article by Burkhard Rost on his secondary-structure prediction program PHD (generally regarded as the most accurate method currently available) and that on local alignment statistics by Altschul and Gish. There are articles (and sections of articles) that provide a clear exposition or overview of the basics of some aspects of bioinformatics, for example the article by Saitou on the reconstruction of gene trees from sequence data. There are accounts of their general approach to problems by expert practitioners, such as the articles by Bork and Gibson and by Koonin, Tatusov and Rudd (in both cases in the area of attributing function to new protein sequence), as well as articles that convey interesting new ideas that will stimulate those writing end-user software to include new features in their programs.

However, it is hard to see how this volume is intended to relate to its predecessor, or to other members of the series. Even as a selective guide to only the most important advances, there are some notable absences. For example, although 'Hidden Markov Model' methods are tantalizingly referred to by several authors, none is described. Artificial neural nets are used in several contributions, but there is no general account of them. There is a suitable article for this purpose in Volume 210 of *Methods in Enzymology*, but it does not appear to be referred to in this one. This is a typical omission – there are other closely related articles in Volume 210, and also in Volumes 202 and 203, but there is no general cross-referencing. The book is very idiosyncratic in its coverage of data service centres – there are articles about EBI and PIR, for example, but not about NCBI or Swissprot. The absence of any reference to Japanese resources is particularly disappointing, in view of the excellent Internet services available from Japanese hosts. Many articles refer, for example, to the 'Smith and Waterman' sequence searching service provided by the EBI, using a MasPar parallel computer and the program MPSRCH, but this version of the program was superseded several years ago and a much better service is provided by the DNA Information and Stock Centre at the National Institute of Agrobiological Resources, Tsukuba, Japan (http://www.dna.affrc.go.jp/). This service, which also carries out BLAST and FASTA searches, has a faster machine, an up-to-date version of MPSRCH and an excellent set of databases, and access

is almost always very rapid. This particular omission may owe something to the long gestation period of a hardback book, but a wider choice of authors (only 2 of the 80–90 are from Japanese institutions) might have alleviated the problem.

The main value of this volume, like its predecessor, is as a reference source for bioinformatics specialists and research workers in the field. Other molecular biologists who turn to it for clear and authoritative help in choosing a method to solve a problem may be left struggling. They will be puzzled, for example, that the chapter on the delineation of protein-coding regions (by Uberbacher, Xu and Mural) makes no reference to the chapter by Staden in the prior volume, although this uses many of the same methods. They will find it hard to reconcile 'The EBI maintains a very high level of quality assurance of the sequence data in the EMBL database' (p. 4) with 'Judging from the poor quality of data bank entries, this scheme apparently fails to detect any but the most obvious errors' (p. 213). In several chapters, they will sink in a flood of unexplained mathematical jargon.

Despite its unevenness, this book is an important addition to the specialized publications in bioinformatics; it is less suitable as an introduction to the field or as a guide to recent developments for a general audience.

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Understanding cytochrome P450

Methods in Enzymology, Volume 272: Cytochrome P450 Part B

edited by Eric F. Johnson and Michael Waterman, Academic Press, 1996. UK£61.00 hbk (468 pages) ISBN 0 12 182173 0

Following the application of molecular cloning techniques, there has been a renewed interest in cytochrome-P450-mediated reactions. P450 enzymes are important in a diverse array of reactions, including drug detoxification in animals, herbicide tolerance in plants and the generation of such biologically active molecules as the insect juvenile hormone and the blue pigments produced in petunia petals. The observation of strong absorption at 450 nm when carbon monoxide is bound to the haem group gives cytochrome P450 its name. In eukaryotes, cytochrome P450s are generally anchored to the endoplasmic-reticulum membrane and receive reducing equivalents from membrane-bound NADPH–P450 reductase, which is used to reductively cleave molecular oxygen, normally producing a hydroxylated organic molecule and water.

Cytochrome P450 Part B will be useful to researchers in the field, providing excellent insights into and know-how of ways of isolating, assaying, cloning and expressing these recalcitrant enzymes in heterologous hosts. There are many step-by-step accounts of experiments performed on P450s from many sources, with a description of the various protocols, results and conclusions. Expression systems are described with full methodologies for holo- and truncated P450s, as well as for P450:P450-reductase fusion proteins produced by manipulation of the DNA encoding these enzymes. These P450 constructs have been successfully introduced and expressed in a wide range of hosts, including *Escherichia coli*, yeast, insects and animal-cell-culture systems. The use of these overexpressed P450s has provided powerful tools for determining the activities of P450s towards a wide range of substrates. Elegant and precise examples of the roles of specific P450s in drug metabolism are recounted, providing insights into why some patients do not metabolize drugs well or develop autoimmune, drug-induced hepatitis. Simple molecular-screening protocols are included that will enable rapid predictions to be made on the ability of a patient to tolerate certain classes of drug. The dramatic increase in understanding of the biology of insect and plant P450s is covered in separate sections, with useful protocols describing approaches to the purification, assaying and cloning of P450 enzymes and sequences. This has particular relevance for herbicide and insecticide tolerance and also for many aspects of secondary metabolism in both plants and insects.

There have been relatively few successful crystallographic studies of P450 enzymes that allow structure–function predictions to be made. However, this text deals in depth with computer-modelling algorithms and with methodologies to improve the chances of crystallizing a particular P450 enzyme. These techniques are described with examples of substrate-fit models and actual experimental data. In the final section, the regulation of P450 enzymes and the generation of transgenic animals with specific disrupted P450s are clearly described, again with in-depth protocols. Perhaps this last section is a taste of things to come from P450 biology, leading to the generation of transgenic organisms with engineered altered activities of specific P450 enzymes. These organisms will undoubtedly be of vast scientific and commercial value in the understanding and exploitation of drug metabolism and pesticide tolerance, and in the generation of valuable secondary products in biological systems.

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New members of the *TIBTECH* board

In this month's issue, *Trends in Biotechnology* would like to welcome seven new members to the advisory editorial board, and also to thank the retiring board members for their tremendous help and support over the years. Our new members are: Terry Coakley, John Durant, Mike Hoare, Lynne Macaskie, James Murray, Chris Sander and Urs von Stockar.

Terry Coakley is a Professor in the School of Pure and Applied Biology, University of Wales, Cardiff, UK.

His research interests are in understanding and exploiting radiation forces on cells and microparticles in ultrasonic-standing-wave fields, and also in understanding the biophysics of local contact formation in membrane–membrane interactions. Applications of ultrasound include: concentrating cells at pressure nodes in a standing wave, facilitating their removal from suspension in batch or on-line flow (nonblocking force-field filter) systems; standing-wave enhancement of droplet coalescence in aqueous biphasic cell or molecule separations, with implications for rapid on-line analytical-scale systems; the exploitation of standing-wave inter-cell and -particle forces in clinical and ecological analytical immunoagglutination of coated particles in the presence of specific cross-linkers. Increasing these applications also leads to the dependence of ultrasonic forces on the properties and concentration of cells and particles being examined.

John Durant is the Assistant Director (Head of Science Communication) at the Science Museum and Professor of Public Understanding of Science at Imperial College, London, UK. In addition, he is the Chair of the European Federation of Biotechnology Task Group on Public Perceptions of Biotechnology, a member of the UK Department of Health Advisory Committee on Genetic Testing and a member of the UK Committee for the Public Understanding of Science. He is also the founding editor of the international journal *Public Understanding of Science*.

He is interested in all aspects of the relationship between science and the public, and his current research interests include surveys of the public understanding of science, public perceptions of biotechnology in Europe and public participation in science and technology policy making.

Mike Hoare is Director of Training and Co-Director of Research within the UK Biotechnology and Biological Sciences Research Council (BBSRC)-sponsored Advanced Centre for Biochemical Engineering and a Professor at University College London, UK. Within the Centre, he coordinates the research in enhanced bioprocess definition and control. He is a Fellow of the Institution of Chemical Engineers (FIChemE, CEng).

His research interests include rapid monitoring, in particular of protein products, heteroforms and key contaminants and downstream processing, especially centrifugation, flocculation, precipitation, crystallization and, more recently, drying of pharmaceuticals.

Lynne Macaskie is at the School of Biological Sciences, University of Birmingham, UK, and has strong collaborations with several European groups.

Her research interests centre on the applications of microorganisms to waste remediation, using immobilized cells or biofilms, and the biochemistry underlying the remediative reactions. Initially, research focused on methods for the removal of metal cations and the development of models to apply the technology economically and realistically. For acidic wastes, interest has focused on the possible applications of acidophilic microorganisms. In some cases, the recovered metallic biomineral has useful properties and a new remediation technology based on this approach, is being developed.

James (Jim) Murray is Professor and Vice-Chairman in the Department of Animal Science at the University of California, Davis, USA.

His research interests include the use of transgenic mouse models to study the genetic regulation of body growth and lipid metabolism based on regulatable growth hormones, the application of transgenic technology to the alteration of the composition and functional properties of milk in mice and ruminants, the development of antisense transgene expression cassettes for tissue-specific regulation of gene expression and the construction of a comparative gene map for the horse.

Chris Sander is involved in research in bioinformatics at EMBL-EBI, the European Bioinformatics Institute near Cambridge, UK, a branch of the European Molecular Biology Laboratory and is Professor of Bioinformatics at the University of Oslo, Norway. He is principal investigator on the GeneQuiz project for the development of an information system for large-scale sequence analysis (and an executive editor of the journal *Cabios*, which will be renamed *Bioinformatics* in 1998). He was instrumental in forming the Department of Biocomputing at EMBL-Heidelberg in 1986 and in founding EMBnet, a network for molecular biologists in Europe, in 1988.

His research focuses on protein structure and function, completely sequenced genomes and intelligent systems for molecular biology.

Urs von Stockar is Professor in Chemical and Biochemical Engineering at the Swiss Federal Institute of Technology, Lausanne (EPFL). He has been a member of the Executive Committee of the European Federation of Biotechnology since 1990 and currently serves as its Chairman. He chairs the Swiss Coordination Committee of Biotechnology, is a member of the Scientific Advisory Committee of the Swiss Academy of Technical Sciences and was Associate Professor at the University of Geneva from 1990 to 1996.

He is interested in integrated bioprocessing, applying high-cell-density cultures, advanced cell-encapsulation methods, hydrophobic membranes and other methods to control culture conditions and for *in situ* product recovery. Other interests include developing advanced, novel on-line monitoring techniques, such as the use of heat-dissipation measurements for monitoring and controlling bioreactors.

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PII: S0167-7799(97)01074-3

Meeting report

Environmental biotechnology: monitoring, mobilizing and mineralizing pollution

It used to be said that the solution to pollution was dilution. Whilst this adage may still have universal applicability, over the past decade there has been an exponential growth in environmental biotechnology research aimed at the amelioration of pollution[1]. Regulatory and governmental interest has focused on the remediation of polluted industrial sites, waters and atmospheres. Much work describing the fate of both organic and heavy metal pollutants in the environment has been published, with proposed remediation strategies involving both chemical and microbiological treatment[2,3]. Research has found commercial application in the monitoring and treatment of industrial waste streams, to satisfy licensing criteria and in waste-water treatment. Usually, such research has been approached from a biological, chemical or process engineering standpoint, with the biological research split between the microbiologists and the geneticists. The *International Symposium on Environmental Biotechnology*★ aimed to bring together scientists working in all these fields, to discuss their recent research in an interdisciplinary environment. Symposium sessions were organized in a generic way, bridging the boundaries between fields such as water, soil and air treatment.

Treatment of waste water

Nutrient removal

The presence of nitrogen and phosphorus compounds in waste-water streams released into the environment leads to many problems. Nitrogenous compounds can be removed by applying aerobic nitrification coupled with anaerobic denitrification. These two processes have been combined in a hybrid biofilm airlift suspension reactor by W. van Benthum (Delft University of Technology, The Netherlands). This process was found to be suitable for the treatment of high ammonia ($>1\ g_N\ l^{-1}$) industrial waste waters. Waste-water treatment systems can be enriched with phosphate-accumulating microorganisms to produce a phosphate-free waste stream. P. Lemmos (Universidade Nova de Lisboa, Portugal) presented work elucidating the metabolic pathway for phosphate removal using nuclear magnetic resonance (NMR) as a noninvasive, nondestructive tool. ^{13}C-NMR and ^{31}P-NMR were used to trace the fate of ^{13}C-labelled carbon substrate *in vivo*, thus enabling the development of a new metabolic model involving glycogen for biological phosphate removal.

Reactor technology

High-rate aerobic waste-water treatment processes that occupy less land and produce less sludge are a focus of research in this field, given impetus by recent and proposed EU directives on sludge treatment and disposal[4–6]. Many such systems have been proposed at a laboratory scale, but T. Stephenson (Cranfield University, UK) restricted his review to viable systems, mostly proved at full scale, and concluded that the economic viability of these systems in comparison with traditional systems has still to be demonstrated. The membrane bioreactor is one example of these high-rate processes that may be coupled to an activated sludge reactor to provide complete retention of sludge (and thus low sludge production). Details of the successful use of this system in the treatment of both raw domestic waste water and high-strength waste water were presented by W. Ghyoot (University of Gent, Belgium). Additionally, the influence of sludge age on cell viability and sludge production rates in a membrane bioreactor was discussed by J. Rols (Institut National des Sciences Appliquées, France).

Biofilter reactors have been used for the aerobic treatment of waste water, their advantages including compactness and the absence of clarifiers. However, regular backwashing is required to remove excess sludge and prevent clogging, necessitating the deployment of expensive equipment. The resulting sludge requires further treatment before handling. E. Daamen (Delft University of Technology) introduced a new type of biofilter reactor, the Biogenerator, aimed at addressing some of these problems. In this biofilter system the waste water passes down through a floating filter, and filter material is continuously removed from the top of the reactor, cleaned and added back at the bottom of the reactor, avoiding the need for backwashing. This system achieved a high chemical oxygen demand removal capacity per unit area, but the removal capacity was limited by biofilm processes, and so research is now aimed at changing the filter material to increase the biofilm surface area.

Kinetics and modelling

As effluent standards for municipal and waste water have become more stringent, the performance of waste-water treatment plants has become a focus for performance evaluation and optimization. To this end, a series of talks on the development and use of models for waste-water treatment processes were presented by X. le Tallec (Research Centre of Compagnie Générale des Eaux – OTV, Maisons-Lafitte, France), H. Temmink (Wageningen Agricultural University, The Netherlands), B. Peterson (University of Gent) and K. Versyck (Katholieke Universiteit, Leuven, Belgium).

Flocculation, granulation and biofilms

Biofilms and flocs (suspended aggregated microorganisms) are responsible for most microbial conversions in natural environments. The applications of biofilms in both the recovery of heavy metals [L. Diels, Vlaams Instituut voor Technologisch Onderzoek (VITO), Mol, Belgium] and the treatment of waste industrial gases (W. Okkerse, Eindhoven University of Technology, The Netherlands) were outlined in detail. Traditional microbiological techniques may not be suitable for the study of biofilm behaviour, because the target populations may be unculturable, and the prevailing microenvironments may differ significantly from the bulk phase. The use of microsensors was discussed by D. de Beer (Max Planck Institute for Marine Biology, Bremen, Germany) and is demonstrably leading to a greater understanding of

★The *International Symposium on Environmental Biotechnology* was held in Ostend, Belgium, 21–23 April 1997.

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PII: S0167-7799(97)01072-X

mass-transfer phenomena in these structures. In parallel to this, S. Zhang (Imperial College of Science and Technology, London, UK) presented work using video imaging to measure biofilm thickness *in situ* and so relate the actual biofilm structure to the diffusion of tracers across the biofilm.

Biodegradation and bioremediation

Degradation pathways

The characterization of degradation pathways at a molecular level is the type of fundamental study that allows the design of remediating biocatalysts, for example, the work of M. Rutter (University of Westminster, London, UK) on the genetic stability of the plasmid-encoded ability to degrade monoaromatic hydrocarbons and H. Smidt (Wageningen Agricultural University, The Netherlands) on the coupling of reductive dehalogenation to halorespiration. K. Timmis (National Research Centre for Biotechnology, Braunschweig, Germany) discussed how organisms can be improved by, for example, assembling a complete degradation pathway into a single strain to avoid the requirement for a mixed culture of organisms, or adding surfactant-producing ability to a strain that degrades hydrophobic contaminants[7]. The production of such bacteria can be approached either by genetic engineering to construct the genome required, or by *in vivo* gene transfer as presented by D. Springael (VITO). This technique has been applied to combine two stages of polychlorinated biphenyl (PCB) degradation, naturally found in separate bacterial strains, into a single strain able to mineralize monochlorinated biphenyls.

Factors influencing the biodegradation process

Having isolated or constructed organisms able to carry out biodegradation, the investigation of factors likely to influence the process in the real environment is required. In marine sediments, the reductive dehalogenation of halogenated aromatic pollutants under methanogenic conditions is well established, but M. Haggblom (Rutgers University, Piscataway, NJ, USA) clearly demonstrated that this important reaction occurs under a variety of other redox conditions.

Pollutants are generally present in the environment together with other contaminants or naturally occurring substrates. Using a conventional substrate-limitation approach, K. Kovárová (Federal Institute for Environmental Science and Technology, Dübendorf, Switzerland) presented findings that may allow us to predict the behaviour of pollutants and the appropriate degrading organisms more effectively. The importance of co-contamination was shown by the work presented by L. Bastiaens (Environmental Technology, VITO). A co-metabolized component of petroleum-contaminated soils (phenanthrene) was found to inhibit the biodegradation of fluorene (a polycyclic aromatic hydrocarbon, or PAH) in both liquid cultures and soil systems, as a result of the accumulation of co-metabolic products.

Conventional wisdom has largely dictated that bioremediation requires transformation or mineralization of the target contaminant. However, recent research has shown that soil binding may in itself be a remediation strategy. J. Bollag (Pennsylvania State University, USA) presented work using ^{13}C-NMR to investigate the fate of cyprodinil (an anilinopyridine fungicide) in soil. Cyprodinil was sequestered (rather than covalently bound) to soil fulvic acid but was covalently bound to humic acid, in which it became cleaved between the two aromatic rings. These bound components were very similar to natural humus, and should not be considered as a hazard. P. Adriaens (University of Michigan, Ann Arbor, USA) built on this theme, suggesting that the apparent recalcitrance of chlorinated aromatic compounds was actually a phenomenon caused by our failure to link contaminant loss with microbial activity, and calling for a stronger integration between environmental analytical chemistry and applied biotechnology[8].

Community structure

The ability to classify bacteria by comparative analysis of ribosomal RNA (rRNA) has enabled new research into microbial community structure *in situ* to be undertaken. Examples of this approach were provided by J. Godon (INRA, Narbonne, France), who had investigated the community structure of a fluidized bed reactor, and R. Amann (Max Planck Institute for Marine Microbiology, Bremen, Germany), who presented work using oligonucleotide probes labelled with a fluorescent dye and targeted to the rRNA of individual phylogenetic groups in activated sludges[9]. This technique avoids the need for the extraction and cultivation of organisms, enabling the identification and monitoring of unculturable organisms, which have been found to be abundant in the environment. Further work aims to link community structure with function through the use of microsensors, and has the potential to improve our understanding of the function of complex microbial communities.

Biosensors

Biosensors consist of a biological signalling component with a signal detection system. Fusion of the *Lux* gene from *Vibrio fisheri* to the promoters of other bacterial genes has allowed the measurement of transcriptional activation in the environment, by measuring the induced luminescence. This approach was used by R. Miller (Oklahoma State University, Stillwater, USA), who studied activation of the *recA* gene (induced in response to DNA damage in bacteria) to measure environmentally induced DNA stress, and by S. Belkin (The Hebrew University, Jerusalem, Israel), who measured oxidative stress using genes induced by peroxides or superoxide radicals. These systems have potential for monitoring processes such as waste-water treatment, measuring the status of habitats *in situ* and studying microbial gene regulation.

Combined waste treatment

The integration of traditionally differentiated biodegradation processes is an important goal if remediation is to be achieved. H. Knackmuss (University of Stuttgart, Germany) discussed an integrated anaerobic–aerobic process for the elimination of electron-deficient xenobiotic compounds. Such compounds can be resistant to initial microbial oxidation, but can be reduced by anaerobic bacteria. Work has therefore focused on novel anaerobic reactions that increase the susceptibility of the xenobiotic to subsequent oxidative catabolism; S. Guiot (Biotechnology Research Institute, Montreal, Quebec, Canada), discussed the dehalogenation of tetrachloroethylene using biofilms integrating aerobic and anaerobic populations.

A process combining physical, chemical and biological processes was outlined by P. Vanneck (Seghers

Engineering Water, Wespelaar, Belgium). Degradation of PAHs with more than four rings is limited by mass-transfer processes, and biodegradation occurs very slowly. Thermal desorption removed this PAH contamination from soil and the removed PAH was subsequently biodegraded in a two-phase silicone-oil and water system.

There is increasing interest in water reuse because of the growing problems of water supply and the increasing cost of disposal of waste water. J. Liessens (Biotim, Antwerp, Belgium) presented case studies of water recycling (use of water for the same purpose as before) and reclamation (use of water for a lesser purpose). Further cleanup of water-treatment effluent is required before reuse, often necessitating filtration through granular activated carbon systems. The resulting water quality and toxicity must be monitored, requiring developments in biosensor technology. Despite these initiatives, public acceptance of water reuse was thought to be low, unless the water had passed through a natural step, for example by being returned to a river.

The bioremediation of a target pollutant can be a function of many variables. This conference demonstrated how far down the road to exploitation some of the techniques are, whether in the field of monitoring, mobilizing or mineralizing. Although successful field application has been limited, a better understanding of biocatalysis, whether alone or as part of an integral process, is developing. Hopefully, progress towards this goal will be further demonstrated by the time of the next symposium, at the turn of the century.

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Workshop

Towards an oral influenza vaccine

Despite the ready availability of commercial influenza vaccines, influenza epidemics remain a major public-health concern. The disease has been referred to as 'the last great uncontrolled plague of mankind', with an average 52 million episodes of influenza-related illness reported each year in the USA alone. From 1992 to 1995, over 120 000 patients died of influenza-related illness in the USA, the majority being over 65 years of age[1]. Clearly, the economic cost of the disease in terms of lost productivity and healthcare provision is considerable.

Deficiencies of current vaccines

So why is it that the existing vaccines are failing to control epidemics and protect the population? This is partly explained by low vaccination rates: it is estimated that 40% or more of high-risk individuals (for example, the elderly and those with chronic pulmonary, cardiovascular or renal diseases or immunosuppression) fail to receive influenza vaccine[2]. In addition, the surface proteins of the influenza virus undergo antigenic 'drift' and 'shift' (see Glossary), mutating into new strains. This means that the formulation of the commercial vaccine must keep pace with the changing virus, and so it is renewed annually to incorporate new strains prevalent in the community. Presently, vaccines contain the surface proteins from two influenza A strains and one B strain, which prime the immune system to resist infection. Predictions of future circulating strains must be made well in advance of the 'influenza season', and occasionally the vaccine (and therefore the antibody responses) do not match the epidemic strain. Finally, the nature of the immune response produced by the parenteral vaccine is restricted. With injectable vaccines, high levels of antibodies to the virus proteins are present in the blood, but the influenza virus first infects the epithelium of the upper respiratory tract and it is at this 'portal of entry' that the first immune defence is required. However, current vaccines are deficient in this area, as little or no secretory antibody or cell-mediated immune responses (required for recovery from infection) are produced at mucosal surfaces. This is the typical host response to infection, which an 'ideal' influenza vaccine should be designed to mimic.

Advantages of oral vaccination

In order to improve the protection rates achieved by vaccination, attention has recently focused on the oral delivery of influenza antigens. By virtue of the common mucosal immune system, it is possible to administer a vaccine orally and achieve an immune response at other, remote, mucosal surfaces, for example the respiratory tract[3,4]. This phenomenon exploits the body's ability to process antigen within enteric pockets of lymphoid tissue, the Peyer's patches, which leads to the dissemination of antigen-sensitized B and T cells to distant mucosal tissues via the efferent lymphatics. However, straightforward administration of inactivated influenza virus or antigen does not produce a satisfactory immune response, at least when using commercially viable doses. A

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PII: S0167-7799(97)01070-6

Glossary	
Antigenic drift and shift	Antigenic drift describes the random point mutations that can occur in the influenza-virus surface antigens, leading to new variants with minor changes in antigenicity. Antigenic shift is a more drastic change in the type of surface antigen caused by the exchange of genes between different strains of influenza A during, for example, a mixed infection with human and avian strains.
B and T cells	Components of the immune response. B cells are lymphocytes that differentiate in the bone marrow and secrete antibodies; T cells are lymphocytes that are concerned with control of intracellular infections.
IgA, IgG	Classes of immunoglobulin (antibody). IgG predominates in the blood, while secretory IgA is the principal antibody in the upper respiratory tract.
Microfold (M) cells	Specialized epithelial cells of the small intestine that overlie the **Peyer's patches**.
Mucosal surfaces	The moist surfaces of the body, including the nasopharyngeal and bronchial surfaces and the gastrointestinal and genitourinary tracts.
Peyer's patches	Defined follicles of lymphoid tissue located along the small intestine.

means of increasing the efficiency of uptake of antigen from the intestine is required, either by specifically targeting the microfold (M) cells that overlie the Peyer's patches or through sustained release of antigen along the length of the intestine. An added complication is the need to protect the antigen from degradation during its passage through the gastrointestinal tract. In addition, to elicit the desired mucosal immune response, the residence time of the antigen within the Peyer's patches needs to be maximized[5]. A number of studies have indicated that the size of the antigen aggregate influences its fate following uptake, with particles of around 5–10 μm diameter becoming restricted to the Peyer's patch[6] and therefore meeting this exacting requirement.

Virus–erythrocyte-ghost vaccine

This has led to the development of a carrier system based on the chicken erythrocyte 'ghost', which has the requisite dimensions. A ghost is derived from the erythrocyte by osmotic shock, which leads to the expulsion of the majority of its cytoplasmic contents, including the haemoglobin. Influenza virus binds efficiently to erythrocytes through a specific ligand–receptor interaction. To assess the immunogenicity of the virus–ghost preparation, intact influenza virus (A/Queensland strain) that had been inactivated by gamma irradiation was first adsorbed to chicken erythrocyte ghosts (Fig. 1).

Mice were immunized orally with a bicarbonate suspension of the

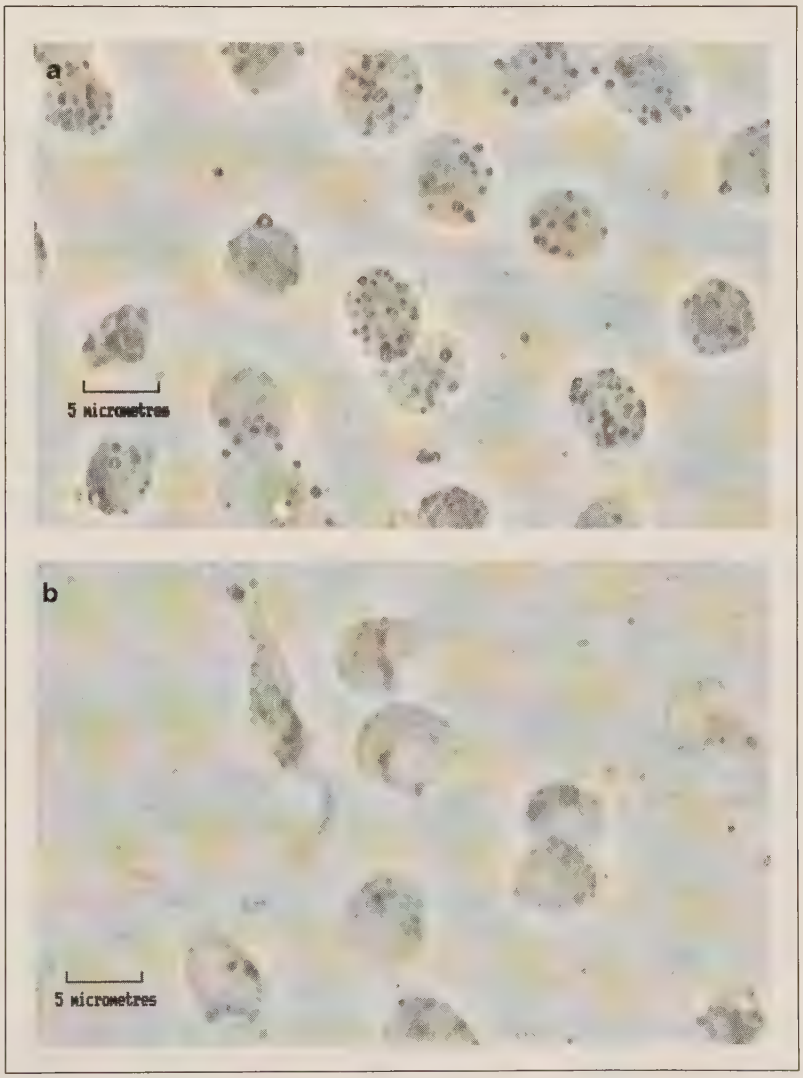

Figure 1
(**a**) Influenza virus bound to chicken erythrocyte ghosts. Virus was visualized by the addition of specific antisera and an enzyme conjugate, which gave an insoluble product. (**b**) Control preparation comprising erythrocyte ghosts with no virus adsorbed.

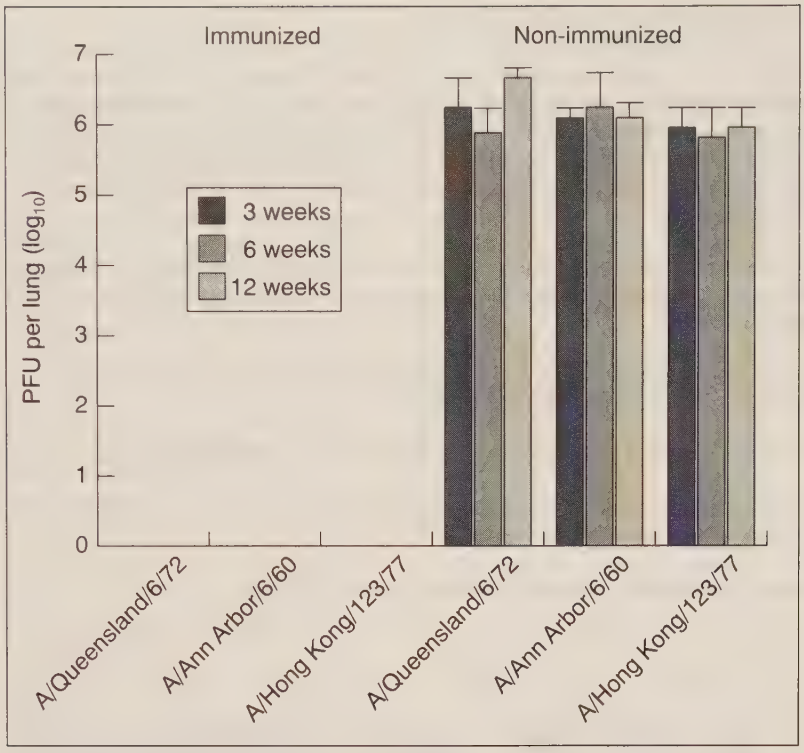

Figure 2

Virus clearance from immunized and control animals following challenge. After administration of the vaccine (A/Queensland strain), mice were left for 3, 6 or 12 weeks before intranasal challenge. The level of virus remaining in the lungs after three days was determined by plaque assay. PFU is the number of plaque-forming units produced by live virus following infection of a susceptible cell line. Clearance of virus by the animal results in the reduction of recoverable virus PFU. A/Queensland/6/72 is an H3N2 subtype, A/Ann Arbor/6/60 is H2N2 and A/Hong Kong/123/77 is H1N1.

complex with different ratios of virus to ghost, or virus alone, on days 1, 3 and 5, and then challenged intranasally with live virus at 3, 6 or 12 weeks following vaccination. For one group, the challenge was with the same strain as that of the vaccine. To determine the degree of cross-protection achieved, other groups were challenged with unrelated strains. The extent of viral clearance was assayed three days after challenge (Fig. 2). In addition, specific antibody levels in lung extracts and nasal washes were determined before challenge (Table 1), and T-cell responses were also measured. No immune response or clearance was observed for mice receiving uncomplexed virus. However, very efficient virus clearance, correlating with high levels of lung IgG and IgA in the upper respiratory tract, was observed in mice given complexed virus and challenged with either the vaccine strain or unrelated strains. A specific T-cell proliferative response was detected and a specific serum antibody response was also raised, as measured by the haemagglutination inhibition test[7,8].

This immunity was found to persist for at least 12 weeks without boosting. The graded dose response studies demonstrated viral clearance and significant levels of IgG antibody in lung extracts and IgA in nasal washes with a total dose of virus of 10^2 $TCID_{50}$ (tissue culture infectious dose, corresponding to the equivalent of 10 ng viral protein). Similar levels of protection were achieved from challenge with various A strains, although this did not extend to an influenza B virus strain. IgA responses appeared to be suppressed at high virus dose, but complete nasal clearance of challenge virus still occurred. Similar patterns of protection have been achieved with different immunizing strains of influenza.

Clinical assessment

At the Australian Institute of Mucosal Immunology, a clinical assessment of the virus–ghost formulation in humans began in December 1996, with a view to enabling an optimal immunization regimen to be defined and establishing safety and tolerance profiles. Analyses are planned to determine whether the findings of the preclinical studies can

Table 1. Antibody response and protection of mice from infection following oral vaccination with erythrocyte ghosts adsorbed to graded doses of inactivated A/Queensland virus

Virus dose in vaccine (log_{10} $TCID_{50}$)[a]	Lung extract IgG (EU ml^{-1})	Lung extract Virus (log_{10} ml^{-1})	Nasal wash IgA (EU ml^{-1})	Nasal wash Virus (log_{10} ml^{-1})
0	4.8 ± 0.2	6.1 ± 0.1	5.8 ± 0.1	4.8 ± 0.2
<1	17.2 ± 0.8	5.4 ± 0.2	10.1 ± 0.6	4.4 ± 0.2
2	67.5 ± 11.0	2.7 ± 0.8	51.6 ± 0.1	< 2
3	65.9 ± 8.5	< 2	42.9 ± 14	< 2
4	65.4 ± 8.4	< 2	14.9 ± 0.5	< 2

[a]Swiss mice were vaccinated on days 1, 3 and 5 and subsequently challenged with live A/Queensland virus on day 19. Assays were conducted three days post-challenge. Antibody levels in serum samples and lung secretions were determined by enzyme-linked immunoassay, and virus titrations by plaque assay. Virus dose is measured in terms of the tissue culture infectious dose ($TCID_{50}$). Antibody titres are expressed as ELISA units (EU), calculated as 100 times the absorbance at a reciprocal dilution of 10 for lung extract and 5 for nasal washing after subtraction from relevant background values. There were five animals in each group, and the values given are the mean ± the standard error of the mean.

be shown to correlate with the clinical immune responses in humans. It is hoped that this will demonstrate that, following vaccination, a wide breadth of protection against different strains of influenza can be achieved, with the induction of mucosal immunity to protect against the initial stages of infection and a cellular immune response to assist in recovery. In addition, it is anticipated that an oral formulation will be more acceptable in the general move away from the use of syringes and needles for the administration of vaccines. Vaccine take-up rates will be raised as safety is improved and adverse effects minimized by vaccination strategies involving mucosal immunization. A greater coverage of the population may be achieved during an epidemic, as vaccine administration will not be necessarily dependent on highly trained staff.

Other developments

Considerable interest has developed recently in the prospects for an oral influenza vaccine, with various methods being employed to improve the uptake of the viral antigens by the M cells. These include the addition of bioadhesive polymers (mucoadhesives), which encourage the adherence and penetration of the antigen through the mucous layer above the M cell[9]. The ability of emulsions and biodegradable-polyester microencapsulation to protect influenza-virus proteins from gastric digestion has also been assessed[10,11]. The presentation of the surface antigens on liposomal surfaces is an alternative option[12,13]. Heat-labile enterotoxin from *Escherichia coli*[14] and cholera toxin[15] have both been found to act as adjuvants for secretory immunity at distal mucosal surfaces in the mouse model. The expression of relevant antigens by recombinant bacterial (e.g. *E. coli*, *Salmonella typhimurium*) and viral (adenovirus and *Vaccinia*) oral vectors have also been explored (reviewed in Ref. 16). The diversity and success of these approaches to oral immunization provide optimism that the mortality and morbidity associated with influenza epidemics may soon be a thing of the past.

Acknowledgements

The technical expertise of the staff at the Australian Institute of Mucosal Immunology at Newcastle, New South Wales is gratefully acknowledged.

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Coming soon to *trends in Biotechnology*:

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An overview of health-biotechnology development in Brazil

Despite the many difficulties shared by all developing countries, there are many possibilities for biotechnological development in Brazil. State-of-the-art biotechnology has enabled important advances in the fields of diagnostic DNA probes and therapeutic procedures to be made. Important partnerships between public and private institutions have been formed, giving rise to new challenges to meet safety requirements and forge new developments in biotechnology[1].

The public-health situation

Within the health sector, recombinant DNA technology will be useful for the control of the main diseases endemic to Brazil, such as malaria, leishmaniasis, trypanosomiasis and dengue, which strike a large percentage of the population. In 1995, according to public-health authorities, Brazil had over 239 000 dengue cases, many of them in the developed south-eastern part of the country, which accounted for 37.9% of the cases. Leishmaniasis, both the cutaneous and visceral (calazar) forms, presented approximately 31 000 cases, with a higher incidence (47.8%) in the north-eastern states. Malaria was the most striking infectious disease in the country, with over 500 000 cases, a very large proportion of which (81%) occurred within the Amazon basin in northern Brazil. The identification of new targets, either mechanisms of cell penetration or specific antigens in the parasite (*Trypanosoma cruzi*), may give insights into the nature of Chagas' disease, which was responsible for over 5000 deaths in 1995. A serological survey of a group of nearly 340 000 blood donors from south-eastern Brazil, showed that more than 3800 were positive for trypanosomiasis[2].

Research on these major public-health problems has increased greatly in Brazil, with increasing use of molecular-therapeutic approaches and the development of recombinant vaccines. The creation of stable vectors and the characterisation and molecular study of peptides from different parasite strains are important projects currently being carried out, mainly with the aim of investigating malaria and leishmaniasis. Other important studies include the development of laboratory trials for the early diagnosis of genetic diseases[3] and the identification of parasites and microbial agents by using enzyme immunoassays and genome mapping with the polymerase chain reaction or restriction-fragment polymorphisms.

The financial situation

The Brazilian government is committed to placing the country amongst the developed nations, but it faces the huge challenges of overcoming great social divides, modernising industry and production systems, and an emerging new economic model[4]. The country has recently become economically stable, with a previous inflation rate of about 30% a month dramatically lowered to 7% a year by 1996. Consequently, as high inflation rates are considered to be under control, the public and private funding of biotechnology can now be sustained for a longer period without severe interruptions, even if the low budget awarded to biotechnology by the federal administration (about 3% of total health investment) is delaying the race for scientific advancement[5,6].

Throughout the 1980s, federal spending on science and technology was approximately 0.7% of the GIP (gross internal product), increasing from US$ 2 billion in 1981 to US$ 3.5 billion in the 1990s. Federal support now exceeds that from private companies and constitutes the main source of funding for science and technology. Today, these funding agencies work together under the supervision of the Ministry of Science and Technology, developing research areas through the establishment of priorities, including the formulation of a special policy for the biotechnological sector.

Scientific activities must adapt to the adverse economic conditions and political regulations. As part of the country's development process, science and technology standards should be promoted by federal intervention, in accordance with the other policies and specific aims of the nation. In Brazil, these challenges will be overcome by the strengthening of an internal structure aimed at increasing the ability to create, acquire and adapt scientific knowledge[8].

The organisational situation

Biotechnological projects within the governmental Brazilian health structure include the *National Programme for Self-sufficiency in Immunobiology*, the *National Programme for Blood and Hemoderivatives* and the *National Scientific and Technological Capacity-building Programme for Biosafety*. These enterprises join several institutions that use recombinant DNA methods to test and develop vaccines and to screen for blood-borne diseases. Another landmark for biotechnology in Brazil is PRONAB (the *National Biotechnology Programme*), initiated in 1981 and aimed at integrating institutions and budgets related to technological activities in the agriculture, energy and health fields. This was the first project to institute plans for building the national capacity for genetic engineering, and is considered highly important due to its complexity and revolutionary impact.

Recently, a governmental programme aimed at teaching the principles of biosafety to laboratory personnel was set up to satisfy the growing demand for biosafety practices, which was caused by the implementation of the *National Programme for Emerging and Re-emerging Diseases*. In order to strengthen the nation's biosafety capability, the Oswaldo Cruz Foundation (FIOCRUZ) organised an *International Course on the Biosafety of Transgenic Organisms in Human Health* and a *Workshop for Specialists in Biosafety* in 1996, sponsored by several local agencies and with the technical cooperation of UNIDO (United Nations Industrial Development Organisation). These events have created a nationwide perception of biosafety principles and their importance in ensuring proper management of the risks of biotechnology in Brazil[9].

The range of subjects covered by this legally binding instrument led the government to set up a framework for the development of suitable guidelines and codes of conduct that involved the main authorities in the health and environmental fields. Brazil has recently established its biosafety regulations and its National Biosafety Committee, which formulates biosafety policies and provides technical advice[10].

The *Convention on Biological Diversity* represented an important milestone in biotechnology. It has put forward regulations for the countries that are parties to the *Convention*

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PII: S0167-7799(97)01059-7

for the Sustainable Development of Biotechnology in Accordance with the Preservation of Biological Species and the Maintenance of Biodiversity, including Brazil, matching issues of biodiversity with core biosafety questions. As a result of a joint effort of the Global Environmental Fund and the Getúlio Vargas Foundation (a private Brazilian centre for financial and economic studies), the Brazilian Biodiversity Fund (FUNBIO) was created at the end of 1996. FUNBIO holds a significant budget for the next two years to support research and development in strategic fields such as agriculture and biodiversity[9].

The legal situation

The Law Project No. 306/95 of the Federal Senate, which deals with controlling access to the biological resources used as raw materials in biotechnological development, will greatly influence national biotechnology and biodiversity, and is currently being discussed at a national level. The Project is also concerned with the protection of knowledge, the rights of traditional communities and the control of technology transfer. This has caused much debate, because it may be hard to determine the source of a given piece of knowledge, especially for traditional practices passed on for many generations. Examples of this situation may be found in the Amazon region, where natives use herbal medicines to treat diseases. If a biotechnological company isolates the active component from a herb by taking advantage of native knowledge, the local population would have royalty rights over the product. Consequently, on becoming law, this Project will have a drastic effect on the study of medicinal plants (estimated to comprise approximately 25 000 plant species, mainly found in the Amazon region). Most are being used in therapeutic preparations and may constitute a potential source of new biotechnological products[11,12].

The Biosafety Law, implemented by the Ministry of Science and Technology through the National Biosafety Committee, has a great impact on biotechnology transfer through its establishment of rules for the proper use of genetic-engineering techniques and the release of genetically modified organisms into the environment. The law also established the National Technical Biosafety Committee. This committee, which is linked to the Ministry of Science and Technology, is composed of eight experts in biotechnology specialising in the fields of human and animal health, plants and the environment. Representatives from the Ministries of Health, Science and Technology, Environment, Agriculture, and Education also participate in the committee[12–14].

Prospects

Investments in advanced biotechnological techniques have increased in Brazil over the past five years in the fields of biological reagents, vaccines, plants, food products and pharmaceuticals. Human insulin, the first Brazilian product developed from genetic engineering, is now consolidated within the market. This shows that the Brazilian biotechnological industry is now organised, due mainly to the work of the Brazilian Association of Biotechnological Enterprises (ABRABI) and the Brazilian Association of Industrial Technology Research Institutes (ABIPT). These associations have a crucial role in influencing public policies for the sector, as well as in coordinating the management and research guidelines for the main Brazilian technology park. The Associations are made up mainly of small enterprises involved in genetic engineering or molecular biology. A number of these companies have formed joint ventures with overseas partners to fund research into infectious diseases, in particular the development of diagnostic kits for the detection of animal and human tropical diseases[4,15].

A peculiar characteristic of the activities involving molecular diagnostics and antigen purification is the convergence of public and private producers, including a few universities and research institutions, within the same market. FIOCRUZ plays a prominent role, followed by the Butantã Institute, the Adolpho Lutz Institute, the Institutes of Biochemistry and Microbiology of the Universities of Rio de Janeiro and São Paulo, and the Centre of Biotechnology of the University of Rio Grande do Sul, which has developed important lines of research in health biotechnology. These institutions have already achieved considerable capacity in the area, arising from the interaction between public and private sectors in research and development[3].

Brazil has a number of institutions that have a long tradition of research in biotechnology and a wide field of application, lacking only the financial strength to supply their needs and achieve full research capacity in biotechnology. Furthermore, there is a shortage of the qualified manpower needed to further the aim of Brazilian health technology – to increase both capacity and autonomy. This situation can only be improved by an integrated effort to set up a national policy for postgraduate studies and a substantial increase in the funding of science and technology.

The development of health biotechnology in Brazil depends on further progress in research, the provision of more and better training programmes, and increased technology transfer. Innovation and competitiveness are also in great demand to increase the rate of development of strategic capabilities. In addition, it will be necessary to implement an appropriate funding policy to enable these technologies to be handled and to increase the sponsoring of researchers and important development projects.

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Transdermal therapy and diagnosis by iontophoresis

Virginia Merino, Yogeshvar N. Kalia and Richard H. Guy

Iontophoresis, the use of an electric current to drive charged molecules across the skin, has the potential to expand the feasible range of drugs for transdermal administration significantly. This method of delivery is being examined carefully with respect to higher-molecular-weight therapeutics (in particular, peptides and small proteins), which cannot be absorbed following oral administration and for which, at this time, an invasive injection remains the only option. In addition, the procedure of so-called 'reverse' iontophoresis would appear to represent a truly noninvasive approach for diagnostic monitoring of blood chemistry.

The topical application of drugs for the treatment of dermatological disease is a logical and long-established part of medical therapy. Equally, the transcutaneous route of drug delivery has been used to alleviate (in particular) pain in the directly underlying tissues, with the obvious advantage of reduced systemic exposure. Transdermal drug administration is, however, more sophisticated, achieving central pharmacological effects. Seven drugs are currently approved in the USA for transdermal delivery – scopolamine, nitroglycerin, clonidine, estradiol, fentanyl, nicotine and testosterone – with many products on the market[1]. Although the first approved transdermal system appeared over 15 years ago, the range of molecules suitable for this means of delivery remains limited. This is due to the excellent barrier function of the skin, in particular the most superficial layer, the stratum corneum[2]. The stratum corneum is cleverly and very efficiently designed to limit the insensible (i.e. in the absence of sweat gland activity) loss of water from the body. At the same time, it serves as a considerable challenge to the pharmaceutical scientist interested in transdermal delivery. Transport of molecules is believed to occur primarily via a tortuous intercellular route, through an essentially lipid-filled matrix. However, passive fluxes of typical, small, organic molecules (even those with reasonably optimal lipophilicities) are low, from a few tenths to perhaps 10 or 20 $\mu g\ cm^{-2}\ h^{-1}$ (Ref. 1). This means that, if a transdermal patch is going to be of a reasonable size, then this route of administration will be limited to only the most potent drugs.

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The role for a transport-enhancing technology like iontophoresis is therefore clear. In addition, the use of electricity to give improved flux offers the highly attractive advantages of control and efficiency of drug use[3]. Not surprisingly, then, the biotechnology and pharmaceutical industries have become interested in the technique for the delivery of, for example, peptide drugs – molecules that are frequently potent but currently impossible to administer by any route other than injection (the gastrointestinal tract being particularly well equipped to digest such molecules completely before they can be absorbed).

However, there are constraints. First of all, one is still talking about potent molecules – for instance, although insulin is deliverable iontophoretically[3], a typical diabetic's daily requirement cannot be met with the technology presently available. Secondly, while iontophoresis in constant-current mode does guarantee control, the efficiency of the process may not be that high. The electrical potential across the skin drives ions through the membrane; the fraction of the total charge passed that is carried by each ionic species depends on their respective ionic mobilities. Needless to say, small, mobile ions such as Na^+ and Cl^- are more efficient at carrying the current than bulky, cumbersome polypeptides. Thirdly, the isoelectric point (pI) of the skin is between 4 and 5, which means that at neutral pH, the membrane supports a net negative charge[4], and thus molecules that remain fully charged between the pI and the physiological pH are most suitable; also, cations are more efficiently transferred than anions because a negatively charged barrier is permselective to positive ions, that is, it will repel anions and so clearly favor cation transport[4]. There is also an

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PII: S0167-7799(97)01069-X

additional electro-osmotic flow of solvent in the direction of cation movement across the skin: as there is preferential passage of positive ions, more of their momentum can be transferred to the solvent in the direction of their electrotransport[5]. This means that, even if a large peptide does not carry a significant proportion of the charge flowing across the membrane, its transport can still be greatly increased by the phenomenon of electro-osmosis[5]. It is generally believed that electrorepulsion falls off significantly with increasing permeant size[6], implying that electro-osmosis may be a major contributor to the mechanism of enhanced peptide delivery by iontophoresis. As discussed below, the convective movement of solvent induced by the imposition of the electric field across the skin provides a unique application of iontophoresis that guarantees its usefulness well into the 21st century.

Therapeutic applications of iontophoresis

Local and subcutaneous effects

Despite a reasonable amount of investigation, the use of iontophoresis for the treatment of local and subcutaneous conditions has been limited. Topically, the most successful applications have been: the relief of palmar and plantar hyperhidrosis[7]; the painless administration of pilocarpine to stimulate sweat flow in the diagnosis of cystic fibrosis[8]; iontophoresis of local anesthetics (e.g. lidocaine) to minimize the discomfort of a planned intradermal or subcutaneous injection or that of a dermatological surgical procedure[9]; and the delivery of steroids and retinoids to treat acne scarring[10]. Nonsteroidal anti-inflammatory drugs (e.g. diclofenac[11] and ketoprofen[12]) have also been given iontophoretically to relieve pain in deeper, subcutaneous tissues and joints. However, at present, the US Food and Drug Administration has approved only one combination drug–iontophoresis system (Iontocaine®).

Systemic effects

Considerable research and development into the systemic delivery of drugs by iontophoresis are in progress. A system for the administration of the potent analgesic fentanyl has entered Phase III clinical trials[13]. However, despite the suitability of iontophoresis for enhancing the transport of many normal drugs that are unable to diffuse passively at adequate rates, research activity has concentrated predominantly on peptide targets. Many such molecules are of sufficient potency, but with their short physiological half-lives and exquisite sensitivity to enzymatic breakdown in the gastrointestinal tract, chronic and repetitive injections are the only option for their administration. It follows, therefore, that delivery through the skin (a tissue of relative low proteolytic activity[14]) is an attractive alternative, assuming that an effective input rate can be achieved.

The electrotransport of many peptides has been achieved, with the majority of work to date performed either *in vitro* (experiments in which the delivery of drug across an excised skin sample is measured) or in animal models. The range of drugs studied is quite diverse, including insulin in various forms[3,15], angiotensin[16], vasopressin[17], several small tripeptides[18–20] and a number of leutinizing-hormone-releasing-hormone (LHRH) analogs[21–25]. The insulin results in small animals have been positive[3], but scale-up to humans presents additional problems[15]. Impressive *in vivo* data for a somatostatin analog[26], calcitonin[27] and leuprolide[23] (an analog of LHRH) have been reported. It has been shown that drug input profiles can be obtained iontophoretically that mimic the profiles produced by either subcutaneous injection or intravenous infusion. The leuprolide study demonstrated a clear pharmacological effect[23].

Obviously, much development work remains to be done, particularly with respect to optimisation of the formulation that will be applied to the skin. Competing ions need to be minimised and electrochemical reactions eliminated. Drug selection also needs to be carefully addressed, because it seems that certain lipophilic cations can associate specifically with sites along the iontophoretic pathway(s). Nafarelin and leuprolide, for example, have hydrophobic 'anchors' in close juxtaposition to their positively charged residue[24,25]. They are capable of a prolonged residence time in the membrane, which (as their concentration increases) leads to neutralisation of the skin's net negative charge, a consequent loss of permselectivity (i.e. the favored transport of cations), a significant decrease in the normal electro-osmotic flow and the inhibition of their own delivery. This has serious practical consequences, not least of which is that maximal delivery is not produced by the highest possible 'driving' concentration. The specificity of the interaction has been unequivocally demonstrated by the synthesis and testing of the tripeptide motif [hydrophobe–hydrophobe–cation] at the centre of nafarelin and leuprolide[28], which produced essentially identical results.

'Reverse' iontophoresis and clinical chemistry

Iontophoresis is a symmetrical process, driving ions in both directions across the skin, both into and out of the body. The potential exists, therefore, to use the technique for extracting 'information' from inside the body; that is, to perform clinical chemistry without blood sampling. The advantages are clear: the procedure is noninvasive and the sample obtained is particle and essentially macromolecule free, the skin acting as an excellent filter. The drawbacks, however, are considerable: for reasonable periods of current passage, the amount of all analytes extracted is small, and thus the generality of the approach is tempered by the necessity for sensitive analytical detection of the target materials in the iontophoresed sample.

A number of applications can be envisaged, including the analysis of general blood chemistry, the detection of diagnostic markers and drug monitoring during therapy. However, it is perhaps the most obvious application that has received the greatest attention and that has advanced most significantly – glucose monitoring in diabetics. It has been unequivocally demonstrated that frequent glucose monitoring and the resultant increased control of blood sugar results in a

dramatic improvement in the morbidity associated with diabetes. The problem is that conventional glucose monitoring is painful, involved and inconvenient; despite much hope for a noninvasive system, none has yet to appear in useful commercial form. However, 'reverse' iontophoresis demonstrates great promise. Despite the fact that glucose is not charged, it is present systemically at sufficiently high concentrations that iontophoretic extraction via the electro-osmotic mechanism is feasible. The principle was first demonstrated *in vitro*[29] and then in a preliminary study in normal human volunteers[30]. Subsequently, an elegant investigation in diabetics experiencing both hyper- and hypoglycemic episodes proved beyond doubt that the method can replace the classic 'finger-stick' approach[31]. The development of a useful system awaits optimisation of the collection formulation (to maximise the extracted volume), perfection of the 'on-board' sensor, validation of the calibration procedure for day-to-day use and verification that skin tolerance to the continuous application of an iontophoretic device is acceptable.

The future of iontophoresis

The progress recorded over the past decade reflects the fact that the renaissance of interest in iontophoresis (a technique known for over a century) is well justified. The high level of enthusiasm has, however, left many basic questions unanswered in the quest for rapid market success. Inevitably, this has resulted in the identification of problems that must now be solved as the technology develops.

(1) Current passage across the skin is limited in terms of its intensity and duration, due to the perception of discomfort and the provocation of skin irritation. A current density of 0.5 mA cm^{-2} appears to represent a ceiling for use *in vivo*[32]. Thus the profile, duration and intensity of current application and their interactions with the local physiology need to be systematically examined.

(2) The formulations to be used at the electrodes must be optimised for drug delivery and stability, electrochemistry and skin compatibility. The balance between the need to reduce competing ions for drug transport and the requirement for ionic species for the electrochemistry and stability of the drug species has to be found[33–35].

(3) Is the mechanism of drug transport primarily electrorepulsive or electro-osmotic? How can this be determined, and how does the mechanism impact upon formulation design and composition? Special attention should be given to lipophilic, cationic drugs that may associate strongly with the iontophoretic transport pathway and completely change the physics of electrotransport.

(4) This preceding phenomenon directs one also to think about drug selection and molecular design. Clearly, given the results that have been obtained for the LHRH analogs nafarelin and leuprolide, the modifications introduced into the peptide structure to improve the pharmacokinetics after injection are not those which would be chosen to optimise the molecule for iontophoresis.

In conclusion, despite the significant challenges remaining, it is a safe prediction that iontophoretic systems will be part of the pharmaceutical 'tool box' of the next century, and that applications for the delivery of peptides, hormones and more conventional drugs will be available. The prognosis for noninvasive glucose monitoring by reverse iontophoresis is also positive, implying that iontophoresis may also play a role in the ultimate development of a smart closed-loop system, the 'holy grail' of drug delivery[36], controlled by a biofeedback mechanism involving the continuous detection of a physiological response.

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focus

β-Ketolases – forgotten hydrolytic enzymes?

Diana Pokorny, Walter Steiner and Douglas W. Ribbons

β-Ketolases are a forgotten group of enzymes that hydrolyse carbon–carbon bonds of 1,3-diketones to give a carboxylate anion and an alkyl ketone. They are ubiquitous and occupy a central catabolic role. We describe the physiological role of β-ketolases and their potential application for kinetic resolution and synthesis. They are compared with heteroatom-bond hydrolases, which are essential to structural and synthetic chemists for the degradation of polymers and the synthesis of single-isomer substances. Will β-ketolases be useful for carbon–carbon bond synthesis?

β-Ketolases hydrolyse carbon–carbon bonds of 1,3-dioxo-compounds. These enzymes are frequently encountered in intermediary metabolism and also hydrolyse 1,5-dioxovinylogous analogues. The first two symposia devoted to enzymic transformations for the provision of single-isomer (homochiral; see Glossary) substances in organic synthesis took place in 1985[1,2]. This field of study has been dominated by hydrolytic enzymes (hydrolases) including lipases, esterases, proteases, amidases, glycosidases and nucleases. They have been ingeniously used for regio- and stereoselective hydrolyses, kinetic resolutions and asymmetric syntheses to give enantiomerically pure substances not easily attainable by conventional chemistry. Hydrolases have also been essential for structural determinations of biological macromolecules, for example proteins, polysaccharides, polynucleotides and phospholipids, and are now essential reagents for specific carbon–heteroatom bond fissions and fusions in chemistry and molecular biology. These enzymes are produced by all free-living organisms. Many are extracellular and stable, and several have well-defined structures as determined by X-ray crystal analysis and, in solution, by NMR studies. Many of the carbon–heteroatom hydrolases are available commercially and are used in various states of purity.

Under appropriate reaction conditions, such as low water activity, hydrolases can catalyse synthetic and transfer reactions. However, there are exceptions, best illustrated by the regio- and stereospecific transglycosylations in polysaccharide biosynthesis[3–5], for example the syntheses of glycogen, amylopectin, dextrans and levans that occur readily in water.

All of these enzymes cleave a carbon–heteroatom bond to give a carboxylate (except the glycosidases, which give an aldehyde) as shown in Box 1. Epoxide hydrolases (EC.3.3.2.3) have more recently become of interest and differ from the rest by catalysing enantio-specific intramolecular hydrolyses to yield *trans*-1,2-diols[6].

In contrast to carbon–heteroatom bond cleavage, carbon–carbon bond hydrolytic cleavage (analogous to a *retro*-Claisen condensation with carboxylate anions) is considered a relatively rare enzymic reaction type, and has been little studied. This is clear from the following evidence: (1) the latest (1992) edition of the IUBMB *Enzyme Nomenclature*[7] lists only ten enzymes that hydrolyse carbon–carbon bonds of β-diketone substrates (EC.3.7.1.1–10) – the previous issue (1984) showed six (Box 2); (2) the symposia held over the past decade have not alluded to β-ketolases; (3) the excellent monographs that have been provided to catalogue biotransformations, after Kieslich's epic volume on microbial oxidations[8], do not mention the β-ketolase enzymes[6,9–11]; (4) the reports of chymotrypsin and trypsin hydrolysing carbon–carbon bonds of suitable β-diketone substrates are not generally recognized[6,9,10].

The physiological role and distribution of β-ketolases

β-Ketolases are very common enzymes in microbial metabolism and in the liver, where they were first described in the hydrolysis of 2,4-dioxoalkanoates and acetoacetone[12]. In mode of action, β-ketolases may not differ much from the carbon–heteroatom hydrolases (Box 1). The methylene carbon situated between two adjacent carbonyls assumes the equivalent of a carbanion structure, and thus resembles the oxygen and

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PII: S0167-7799(97)01055-X

Glossary

β-Diketones – substances possessing 1,3-dioxo functionality in the carbon chain.

Vinylogous β-diketones – these are 1,5-dioxo compounds with a double bond in the 2,3- or 3,4-positions. It is likely that under physiological conditions their aqueous solution structures are represented by resonance-hybrid distributions of the π electrons between carbons 2 and 4 (as shown by the dotted bonds in Box 2). Additionally, enolate and hydrate forms occur in aqueous solution.

Regioselectivity – preference for position in the substrate molecule undergoing reaction.

Stereoselectivity – the preferential selection or formation of an asymmetric centre for enzyme catalysis, or the orientation of substrate binding.

Kinetic resolution – the discrimination of one optical isomer of a racemate during reaction.

Asymmetric synthesis – the formation of one or more new asymmetric centres in chemical or enzymic reactions. The products are usually called **single isomers**, or **homochiral**. It should be noted that an asymmetric synthesis with a substance that contains more than one optically active centre may lead to either a single-isomer product or a mixture of diastereomeric species.

Heteroatom – an atom other than C or H, e.g. N, O, S.

***meta*-Ring cleavage** – this refers to the enzymic incorporation of both atoms of O_2 into a substrate (catechols, quinols, hydroxyquinols) with ***extra*-diol** regio-specificity; this leads to a carboxylate and a carbonyl by carbon–carbon bond cleavage in the products.

***extra*-Diol** – ring cleavage occurs between a carbon bearing a hydroxyl group and an adjacent carbon that does not.

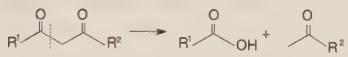
Baeyer–Villiger reactions – the chemical or enzymic transformation of a ketone to an ester or lactone. The usual chemical reagent is a peracid, whereas enzymes insert one atom of molecular oxygen between the carbonyl and an adjacent carbon atom.

Claisen condensation – a classical chemical reaction for the synthesis of carbon–carbon bonds from an ester and aldehyde or ketone precursors.

Homochiral – describes single asymmetric centres and is equated with **single isomers** by some authors.

Single isomer – a pure molecular species that could possess one or more isomeric forms.

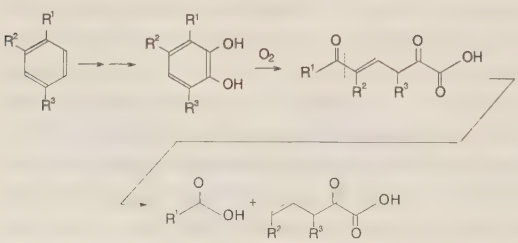
nitrogen in esters, anhydrides and amides by providing an electron pair for the formation of the methyl ketone and carboxylate products (Eqn 1):



(1)

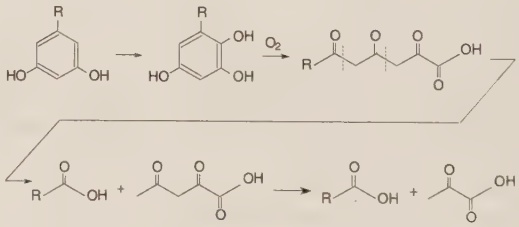
Like the carbon–heteroatom hydrolases, the physiological role of the β-ketolases in microbial and mammalian catabolism seems clear. Their quantitative importance in carbon–carbon bond cleavage is difficult to dismiss. Substrates for carbon–carbon bond cleavage include:

(1) the ubiquitous families of vinylogous 1,5-diketones that are formed after dioxygenative *meta*-ring cleavage of arenes[13–20] (Eqn 2):



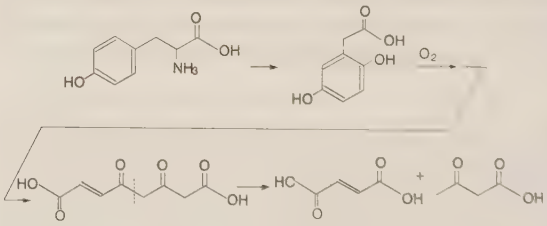
(2)

(2) the 1,3-di- and 1,3,5-triketones formed from *extra*-diol dioxygenative cleavage of quinols, hydroxyquinols[21–24] and safroles[25] (Eqn 3):



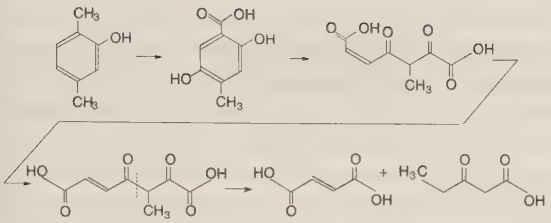
(3)

(3) fumarylacetoacetate and acetoacetate formed from phenylalanine and tyrosine[3,4] catabolism (Eqn 4):



(4)

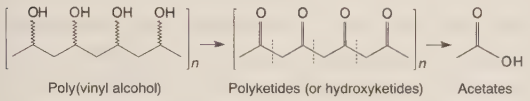
and gentisates from some xylenols[26,27] (Eqn 5):



(5)

and (4) the 1,3-dioxo-intermediates formed during aerobic and anaerobic catabolism of mono- and bicyclic alicyclic compounds[19,28] and some alkaloids (atropine)[20] (Box 3).

The synthetic polymer polyvinyl alcohol, which has only carbon–carbon bonds in the main chain, is biodegradable by microbial oxidation of the alternating secondary alcohol groups to give β-diketones that are hydrolysed by β-ketolases (Eqn 6). Both the oxidases and hydrolases that degrade these polymers are extracellular enzymes[29–31], whereas the β-ketolases mentioned before are intracellular.

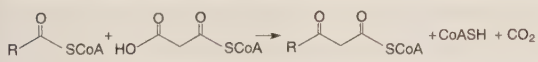


(6)

Potential value of β-ketolases for synthesis

β-Ketolases are not available commercially, and therefore, unlike carbon–heteroatom hydrolases, have not been used by organic chemists for synthesis (for example, for kinetic resolutions or the reversal of the hydrolytic equilibrium in low-water-activity solvents), despite their potential. Nevertheless, the β-ketolase group of enzymes, and their vinylogous counterparts, are possibly as widespread as other hydrolases, and could conceivably be useful for the production of polyketides.

Natural products derived from the various polyketide and chorismate enzymic routes of biosynthesis may represent the most diverse group of natural compounds in terms of their chemical structure and biological function. A summary of polyketide biosyntheses by the *Actinomyces* group of bacteria[32] shows the value of combining genetics with chemistry. These biosynthetic reactions, catalysed by synthetases using thioester substrates, are driven towards synthesis by concomitant decarboxylation of the donor malonyl thioester and its addition to the growing carbon β-ketide chain (Eqn 7).



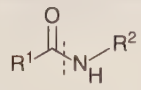
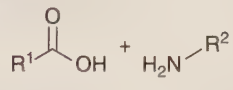
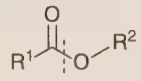
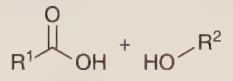
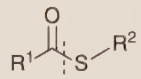
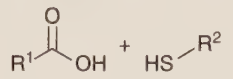
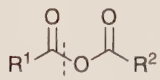
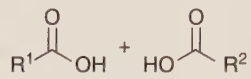
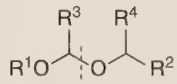
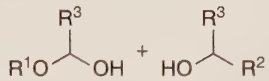
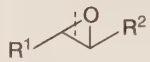
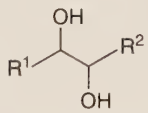
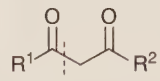
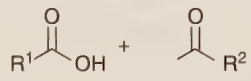
(7)

The variety of products formed is dependent on chain elongation with aceto-units, similar in principle to the carbon–carbon bond formation in fatty acid biosynthesis. The exquisite specificities of the enzymes involved allow stereospecific modifications of the growing β-oxochain. The incorporation of branched chains, by using alternative thioester precursors at precise positions in the extension process, gives stereochemically discrete asymmetric centres. Reductases then provide secondary alcohols. Aromatase enzymes and other cyclases catalyse the formation of ring structures from these aliphatic polyketides, leading to many compounds of biological and pharmaceutical interest such as antibiotics (erythromycins, tetracyclines), feed additives (monensin, avermectin) and anticancer agents (doxorubicin)[32].

Catabolic reaction sequences, found mostly in microorganisms, use enzymes to reverse these biosynthetic processes by hydrolysis as an essential part of the carbon cycle. The provision of 1,3-dioxo-compounds (or their vinylogous 1,5-dioxo and 3-amino-1-oxo analogues) by enzymes (Box 2) is an essential feature for easy carbon–carbon bond cleavage, and is readily achieved by decarboxylases, aldolases and some transferases[3,4].

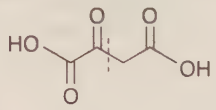
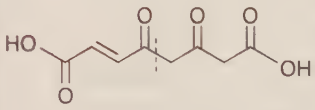
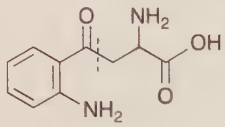
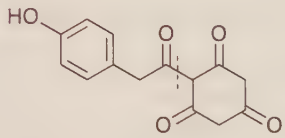
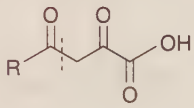
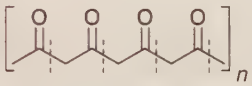
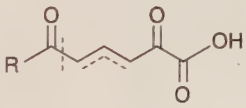
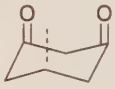
β-Ketolases are involved in diverse reaction pathways; particularly noteworthy is their ability to cleave branched-chain β-diketones, as in the thymol catabolic pathway of *Pseudomonas putida*[23]. However,

Box 1

Substrate type	Products	Enzymes (generic)
		Proteases Peptidases Amidases
		
		Lipases Esterases
		
		Glycosidases
		Epoxide hydrolases
		β-Ketolases

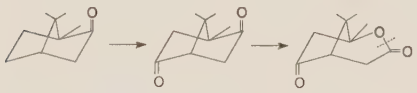
Examples of hydrolytic enzymes, with generic names to indicate their physiological roles. It should be recognized that many of these enzymes catalyse the hydrolysis of other substrate types, for example, proteases hydrolyse suitable esters, thioesters, anhydrides and β-diketones. Other examples of hydrolytic enzymes, such as nucleases, phosphatases, sulphatases, glucosidases, are listed in the International Union of Biochemistry and Molecular Biology (IUBMB) *Enzyme Nomenclature* (1992) treatise.

Box 2

Substrate	C–C bond hydrolase	EC number
	Oxaloacetase	3.7.1.1
	Fumarylacetoacetase	3.7.1.2
	Kynureninase	3.7.1.3
	Phloretin hydrolase	3.7.1.4
	Acetopyruvate hydrolase (Acylpyruvate hydrolase)	3.7.1.5(6)
	β-Diketone hydrolase	3.7.1.7
	2,6-Dioxo-6-phenylhexa-3-enoate hydrolase (2-Hydroxymuconate-semialdehyde hydrolase)	3.7.1.8(9)
	Cyclohexane-1,3-dione hydrolase	3.7.1.10

β-Ketolase enzymes listed in the IUBMB *Enzyme Nomenclature* (1992). Only the trivial or original names are given in column 2.

when γ-diketones (1,4-diketones) are formed, as in camphor metabolism by *Pseudomonas* and *Mycobacteria*, then Baeyer–Villiger lactone-forming enzymes are responsible for the carbon–carbon bond cleavage. Thus metabolic pathways that yield 1,4-diketones do not include carbon–carbon bond hydrolysis reactions. The carbon chain is broken by oxygenation to prepare it for carbon–heteroatom hydrolysis:



(8)

The stereochemical course of β-ketolase reactions that use chiral substrates is not known, but this seems of little importance because the labile protons of the methylene carbon readily allow non-enzymic epimerization of these carbon centres.

Synthesis of poly-β-ketides therefore may seem to be a futile exercise for the synthesis of single isomers but nature has accomplished this in many ways, producing a great diversity of chemical structures with various biological functions. This epimerization of stereoisomers may limit the use of β-ketolases for stereochemical syntheses at methylene-carbon centres, but should not affect their use for enzymic discrimination of nearby asymmetric atoms. However, single isomers might readily be obtained by selective reduction of β-diketones with substituted intervening methylene groups or by other chemical modifications.

Two β-ketolases have been successfully purified to homogeneity: those acting on acetopyruvate (EC.3.7.1.5)[22] and fumarylacetoacetate (EC.3.7.1.2)[33] (Eqns 2 and 3).

Chemical and enzymic hydrolysis of acetopyruvate

Three different carbon–carbon bond cleavages have been proposed to occur during acetopyruvate hydrolysis. The first suggestion was made by Krebs and Johnson[34], who proposed that decarboxylation of acetopyruvate was responsible for the appearance of 'ketone bodies' during the formation of acetoacetate, but this has not been substantiated by subsequent evidence. Lehninger and Witzemann[35] then reported that oxalate and acetone were the only products of alkaline hydrolysis after formation of the dianion, enolate–carboxylate. They attributed the carbon dioxide evolution observed by Krebs and Johnson[34] to the bicarbonate buffer that had been used for the enolization reaction. Finally, Davey and Ribbons[22] reported that the enzymic hydrolysis of acetopyruvate gave equimolar quantities of acetate and pyruvate[21,22]. These suggestions are summarized in Fig. 1.

The structure(s) of acetopyruvate in aqueous solution is quite complicated and pH dependent. This was convincingly shown by Guthrie[36], who demonstrated by ^{1}H-NMR (continuous wave) experiments that three main structures occur in equilibrium and their distributions are pH dependent, a feature also seen later by UV spectroscopy[22] and one-dimensional ^{1}H-NMR-FT spectroscopy (Fig. 2).

Acetopyruvate hydrolases have been purified to apparent homogeneity from beef liver[33,37] and from *P. putida* strain 01 grown with orcinol[22]. Physical-chemical data were not provided for the liver enzyme, but the enzyme from *P. putida* is reported to be a single polypeptide of approximately 38 kDa, induced by growth with resorcinylic compounds[21,22,24]. Acetopyruvate was the only substrate found for the bacterial enzyme[22], although many higher homologues were also substrates for the enzyme purified from liver[12,33,37]. Several analogues, such as fumarylacetoacetate, acetoacetate and 2-hydroxymuconic semialdehyde, were not hydrolysed by either enzyme. Oxalate, oxaloacetate and acetoacetate were competitive inhibitors of both acetopyruvate hydrolases[22]. It is possible that

the acetopyruvate hydrolase from *P. putida* is also responsible for the two sequential hydrolyses of the tri-β-ketides that are formed initially by dioxygenative ring cleavage of the hydroxyquinol substrates formed from resorcinols[21,24] and thymol[23].

Another study of the acetopyruvate hydrolase purified from liver indicated that its physiological role might be the hydrolysis of fumarylacetoacetate[37]. This product is formed after dioxygenative ring fission of homogentisate and isomerization of maleylacetoacetate (Eqn 4), which are central reactions in phenylalanine and tyrosine catabolism[3,4].

In 1961, Hayaishi and co-workers[38] proposed acetopyruvate as an intermediate in a *meta*-ring cleavage pathway for catechol by a pseudomonad, although this has not been substantiated. A hydration–aldolase sequence[14,16] has become the accepted pathway for the formation of pyruvate from arenes after hydrolysis of vinylogous β-diketones; this is supported by enzymic analysis, as well as by protein and gene sequence data[17]. The catabolic sequence used by a *Pseudomonas* sp. for the catabolism of safrole(s)[25] does appear to use a β-ketolase pathway. It was concluded that the formation of valerates from 4-propyl- and 4-propenylcatechols and their aldehydes was neither detected nor oxidized by cell extracts in the presence of NAD(P).

Box 3

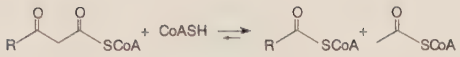
Growth substrates for biodegradative pathways	Substrates for β-ketolases
Orcinol	2,4,6-Trioxoheptanoate
Resorcinol	2,4,6-Trioxoheptanoate
Thymol	3,7-Dimethyl-2,4,6-trioxo-octanoate
Xylenols, Gentisates (e.g. Eqn 5)	Maleylpyruvate Fumarylpyruvate
Safroles	2,4-Dioxooctanoate
Atropine	1,3,5-Trioxocycloheptane

β-Ketolases described for intermediates in bacterial catabolic pathways. Column 2 shows the variety of β-ketides formed during the catabolism of the compounds in column 1.

Enzymic hydrolysis of vinylogous β-ketones

When 3-substituted catechols (which are formed from many arenes) undergo *meta*-ring cleavage, vinylogous β-diketones are produced that are hydrolysed to vinylpyruvate(s) and a carboxylate [hydrolases EC. 3.7.1.5(8,9); Eqn 2]. The equivalent product from catechol itself, 2-hydroxymuconic semialdehyde, also undergoes enzymic hydrolysis (hydrolase EC.3.7.2.9), but this may have little physiological significance because dehydrogenases provide oxalocrotonate(s) for decarboxylation to give vinylpyruvates[17], and the hydrolysis rates of the aldehydes are low compared with those of the ketones. This conclusion is reinforced by the experiments of Harayama *et al.*[39]

Several vinylogous β-ketolases have been purified and they show a variety of subunit structures and substrate specificities. Data are not yet available to describe the catalytic mechanisms of these hydrolases; such studies depend on a knowledge of the equilibrium-solution structures of the substrates (see Ref. 36 and Fig. 2). Solution structures have been deduced from UV–visible spectroscopy, but need to be substantiated by ^{1}H- and ^{13}C-NMR *in aqua*. Recently, Diaz and Timmis[40] have identified (by DNA sequence analysis and site-directed mutagenesis) the catalytic residues of the *xylF* gene product [hydrolases EC.3.7.1.8(9)] from the lower pathway operon of the TOL plasmid from *P. putida* pWWO[17], and these were similar to those deduced for an isofunctional hydrolase from *P. putida* F1 encoded by the *todF* gene[41]. The familiar Ser-His-Asp catalytic triad, found in the same primary amino acid sequences of the mammalian serine proteases and the carboxylesterases of various prokaryotic and eukaryotic sources, was present in the reported sequence[39,40]. It remains to be seen whether other β-ketolases are similar or are of the subtilisin-type serine enzymes and whether they use a cysteine nucleophile or are metallo-enzymes (as might be suggested from the divalent-metal-ion-stimulated acetopyruvate hydrolase[22]). Two cysteine residues in the active site are clearly involved in both half-reactions of the aceto-acetyl-CoA thiolase cleavage reaction for carbon–carbon bond cleavage, one of which is acetylated and the other of which deprotonates the second acetyl-CoA partner in this *retro*-thio-Claisen condensation[42] (Eqn 9):



(9)

Conclusion

Although the carbon–carbon bond hydrolase enzymes have been known for five decades, there have been very few enzymic or biochemical studies carried out on this group of enzymes. However, the advances made in the past 20 years with carbon–heteroatom hydrolases in order to understand (1) their crystal and solution structures, (2) their regio- and stereoselectivities for the provision of single-isomer substances from racemates, *meso*- and prochiral substrates, and (3) their reversibility for synthesis in low-water-activity solvents, are both extensive and remarkable. Similar studies are necessary to examine the value of β-ketolases for kinetic resolutions and for asymmetric syntheses with carbon–carbon bond formation in organic solvents.

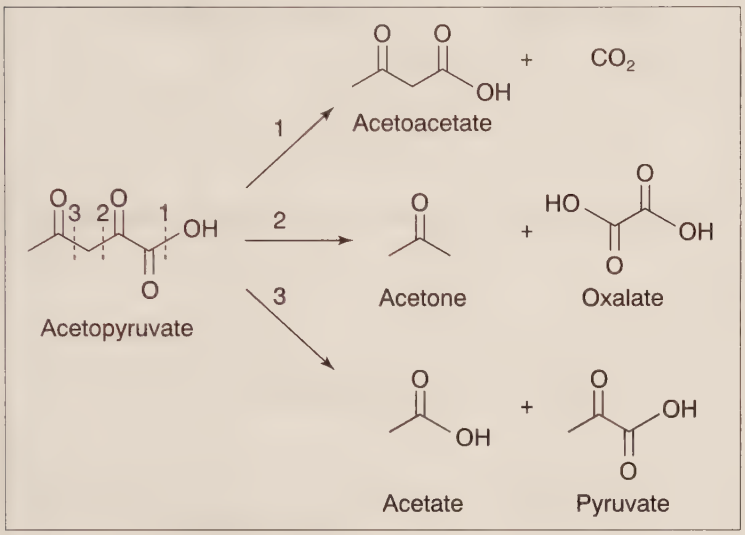


Figure 1

Carbon–carbon bond cleavage reactions proposed for acetopyruvate: (1) Krebs and Johnson[34]; (2) Lehninger and Witzemann[35]; (3) Davey and Ribbons[22].

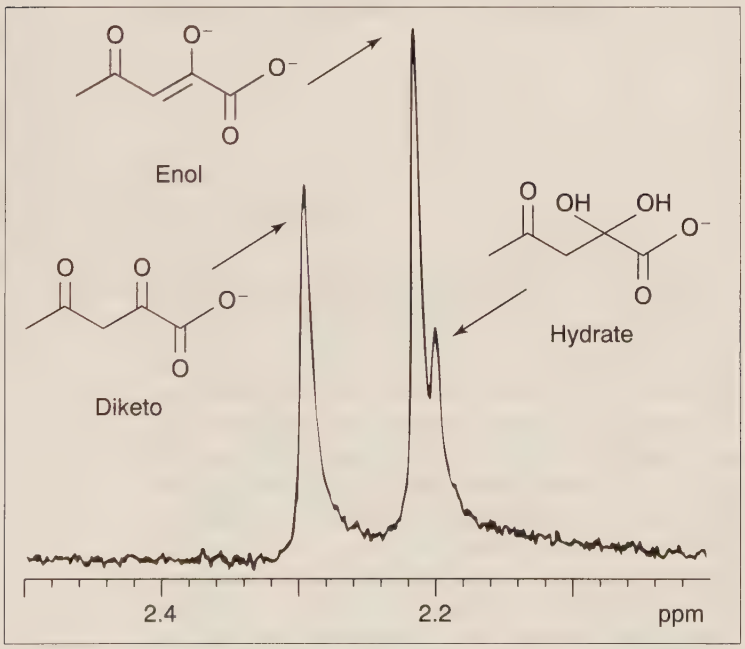


Figure 2

^{1}H-NMR signals of the methyl groups of acetopyruvate observed in aqueous solution at pH 7.5. These are attributed to diketo, enol and hydrate structures[36].

Acknowledgements

Grants from SFB Biocatalysis (Austrian Science Foundation) and National Bank (Austria) supported this work. Jörg Weber (Graz) provided the ^{1}H-NMR spectrum shown in Fig. 2.

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Integration of foreign DNA and its consequences in mammalian systems

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The insertion of foreign DNA into the genomes of established cells and organisms and the consequences of this integration event are of significance for viral oncology, reverse genetics, transgenic organisms, human somatic gene therapy and evolution. This review summarizes recent experimental findings and focuses on the alteration of cellular DNA methylation at regions remote from the site of insertion. We also discuss experimental data demonstrating that foreign DNA ingested by mice is not completely degraded in their gastrointestinal tracts; fragments of this DNA have been found to be covalently linked to DNA with 70% homology to the mouse IgE receptor gene.

The genomic fixation of foreign DNA in mammalian systems has been studied in our laboratory for many years[1,2] and can be considered to be a mechanism of general biological significance, for example in the oncogenic transformation of cells by human adenoviruses. Apparently, mammalian and other eukaryotic cells are capable of transporting foreign DNA to their nucleus and integrating it into their own genome. However, the mechanism of this insertion and the possible defence systems competing with it are not well understood.

In many experiments in reverse genetics, the fixation and expression of foreign genes in eukaryotic cells, upon transfection into the cell, depend on the efficiency of these genomic insertion events. Thus the development of workable approaches to human somatic gene therapy will have to cope with the problems and consequences of foreign DNA integration before useful regimens for the long-term treatment of disease become available. In addition, discussions about the evolution of organisms have raised the issue of the transfer of genetic material from one organism to another. Our findings on the persistence of DNA fragments from the food supply in the gastrointestinal (GI) tract of mice, the uptake of such fragments into different organ systems of the animals and the linkage of foreign DNA fragments to mouse-like DNA[3] have lent support to arguments about the feasibility of the occasional, if rare, acquisition of foreign DNA by established organisms.

The results on integrated adenovirus type 12 (Ad12) or bacteriophage λ DNA into hamster genomes led us to investigate the consequences of inserting foreign DNA into the mammalian genome. These consequences may be summarized as follows.

(1) The integrated foreign DNA often becomes *de novo* methylated in specific patterns, causing the long-term silencing of foreign genes[4,5].

(2) In segments of the recipient genome that can be located *in trans* and remote from the site(s) of foreign DNA integration, marked alterations in the patterns of cellular DNA methylation have been observed (Ref. 6; K. Kämmer *et al.*, unpublished). Due to the fact that levels of DNA methylation are frequently inversely correlated with eukaryotic gene expression[7], alterations of cellular DNA methylation are likely to be associated with changes in cellular gene-transcription patterns. Such changes would have severe consequences for cell function.

(3) Insertion of foreign DNA into an established genome can also lead to insertional mutagenesis (e.g. by deletions of cellular genetic information at the site of integration). However, we submit that the new functional type of insertional mutagenesis due to alterations in cellular DNA methylation and expression patterns is possibly of greater importance[1,2].

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PII: S0167-7799(97)01061-5

Integration of foreign DNA

Several parameters of the integration phenomenon studied in our laboratory model systems are summarized in Box 1. These observations and conclusions have been derived from the adenovirus–mammalian-cell system (for recent reviews covering similar topics in plant systems see Ref. 9), and suggest that the integration of foreign DNA seems to obey similar rules in many different eukaryotic systems. It is possible that the mode of foreign DNA transfer into eukaryotic cells – whether it is by viral infection, various transfection protocols, injection or another method – does not affect the outcome of the integration reaction or its characteristics although this is not yet directly supported by the results of an extensive series of comparative investigations. We also surmise that the mechanisms of foreign DNA integration are very flexible and can cope with many different partners in integrative recombination. Figure 1 demonstrates the integration of Ad12 DNA into the hamster cell genome at both the chromosome and nucleotide-sequence levels.

Other systems

Within the confines of a short survey, it is impossible to do justice to much stimulating work from other laboratories working on different systems. The reader may be interested in a few recent examples studying the integration of DNA of an adenovirus–SV40 hybrid[10], adeno-associated virus[11], human papillomavirus[12] or retrovirus[13]. Some of these reports

Box 1. Elements of foreign (adenoviral) DNA integration into established mammalian genomes[a]

1. Foreign DNA is integrated in multiple copies (1 to >30), frequently in pseudotandem arrays. Rearranged viral DNA or cellular DNA may be located between neighbouring viral genomes.
2. Free viral DNA has never been found to persist in Ad12-transformed hamster cells or in Ad12-induced hamster tumour cells.
3. Depending on the permissivity of the cell type harbouring viral genomes, the entire Ad12 genome or fragments of viral DNA persist in an integrated state. Hamster cells are completely non-permissive for the replication of Ad12 DNA; hence in Ad12-transformed hamster cells, intact viral genomes are integrated. Ad2 or Ad5 can replicate in hamster cells, and these cells carry only fragments of these viral DNAs.
4. At the termini of the integrated viral DNA, viral nucleotides can be missing – up to 174 nucleotides have been found to be deleted in one example. In other instances, not a single viral nucleotide has been lost.
5. Similarly, at the cellular site of viral DNA insertion, again, from nothing to several kilobases of cellular DNA may be deleted.
6. There is no evidence for adenovirus DNA integrating at specific cellular sites. Many different loci of integration have been identified by both the fluorescent *in situ* hybridization (FISH) technique or by determinations of the nucleotide sequences at numerous junctions between viral and cellular DNAs. In one series of experiments, 60 different Ad12-induced tumours were analysed, and the regions of Ad12 DNA insertion were all found to be different. However, one cannot state that adenovirus DNA integration is random; there could be selective sites.
7. At the sites of linkage between the cellular and foreign DNAs, patchy nucleotide-sequence homologies have very frequently, but not always, been observed. These incomplete homologies can span up to 10–20 nucleotides, and have been found in neighbouring cellular and viral sequences and even between the persisting sequence of one of the recombination partners and the deleted sequences in the other partner.
8. Computer-aided analyses of the nucleotide sequences at the junction sites suggest the possible formation of stem-loop structures.
9. The integrative recombination between foreign Ad12 and mammalian cellular DNAs is probably akin to non-homologous recombination, although this classification does not account for all the observed features of the reaction, for example the patchy homologies between viral and cellular nucleotide sequences.
10. The cellular nucleotide sequences at or close to (within a few hundred nucleotides of) the site of integration have often been found to be transcriptionally active. Could regions of cellular transcriptional or other activities and the chromatin structure characteristic for such areas predispose the cellular site for its recombination with foreign DNA?
11. The integrated foreign DNA is frequently *de novo* methylated. This process is initiated at certain, hitherto poorly characterized, sites in the foreign genomes and subsequently spreads across large segments of the integrated foreign DNA. The site of foreign DNA insertion and its structure seem to play an important role in the initiation of this *de novo* methylation reaction. Nucleotide sequences in the integrates may also contribute.
12. A second important consequence of foreign DNA integration into an established mammalian genome is the alteration in patterns of DNA methylation that can occur over extensive regions of cellular DNA remote from the insertion site. The locations of the cellular genome segments thus affected may well depend on the region of foreign DNA integration.
13. The integration of foreign DNA into mammalian cells is in general very stable over many continuous cell generations. However, in isolated instances, the foreign DNA can be lost partly or *in toto* by unknown mechanisms. Could this be the reversal of the insertion event?
14. The insertion of genomes of the oncogenic viruses can be associated with the transformed or the oncogenic phenotype. It is very likely that viral gene products and their specific interactions with cellular proteins, for example tumour-suppressor-gene products, play an important role in the transformation mechanism. However, we favour the possibility that a novel type of insertional mutagenesis, arising from extensive changes in cellular DNA methylation patterns after foreign DNA insertion, can contribute to the generation of the oncogenic phenotype of the cells.

[a]The findings summarized here may have general validity for the integration of many different types of foreign DNA in mammalian systems, but we wish to emphasize that these results have only been documented in detail for Ad12 DNA, and in part for bacteriophage λ DNA, in hamster cells. The data apply to integrated DNA transferred to the cell nucleus either by virus infection or by DNA transfection. For recent reviews see Refs 1, 2 and 8.

examine the role of viral DNA integration in oncogenesis[12] or the stable fixation of foreign DNA in the mammalian genome[11,13].

De novo methylation of integrated foreign DNA – a cellular defence mechanism?

In both cultured cells transfected with foreign DNA and transgenic organisms, the newly integrated foreign DNA frequently becomes *de novo* methylated. This topic has been recently reviewed[5,9] and will not be treated in detail here. The *de novo* methylation of foreign DNA integrates can be considered a ubiquitous phenomenon[9] and so it has been proposed that *de novo* methylation constitutes a cellular defence mechanism to silence integrated foreign DNA or genes[14]. Once all other defence systems against the insertion of foreign genes in a cell or organism have failed, a final silencing mechanism at the level of foreign DNA integrated into the host's genome can be selectively applied. Thus, the expression of most, but not necessarily all, foreign genes can be prevented.

Of course, this defence mechanism turns out to be problematic for the generation of transgenic organisms and for some of the goals of human somatic gene therapy. Methylation, and the ensuing gene inactivation, might thwart the aim of long-term foreign gene expression in some of the experimental schemes on which plans for the generation of transgenic organisms and gene therapies are based.

Alterations of cellular methylation patterns subsequent to foreign DNA insertion

We have shown that the insertion of Ad12 DNA into the hamster genome and the transformation of these cells by Ad12 can lead to marked increases in the levels of DNA methylation in several cellular genes and DNA segments (Ref. 6; K. Kämmer *et al.*, unpublished). Because such alterations in cellular DNA methylation patterns are likely to affect the transcription patterns of cellular genes, it is conceivable that the changes in cellular DNA methylation patterns have played a role in the generation of the Ad12-transformed phenotype. We have described these alterations as a new functional type of insertional mutagenesis[1,2].

We have also generated clonal BHK21 hamster cell lines that carry in their genomes integrated bacteriophage λ DNA, usually in a single chromosomal location as determined by the FISH (fluorescent *in situ* hybridization) technique. In different clonal cell lines, the loci of foreign DNA insertion are different. The integrated bacteriophage λ DNA becomes *de novo* methylated. In a number, but not all, of the λ-DNA-transgenic hamster cell lines generated, the levels of DNA methylation in the retrotransposon genomes of the intracisternal A particles (IAP)[15] are increased in comparison to the non-λ-DNA-transgenic hamster cells (Ref. 6; K. Kämmer *et al.*, unpublished). The possibility that the BHK21 hamster cell genomes might represent mosaics of DNA methylation patterns in the IAP segments has been considered, and shown to be unlikely by demonstrating that, in the DNA of a total of 78 individual BHK21 cell clones, the IAP methylation patterns are identical. These patterns are also identical to those in the original BHK21 cell population.

Figure 1
Chromosomal DNA integration of Ad12 DNA into the hamster cell genome in the Ad12-transformed hamster cell line T637. (**a**) Chromosomal location of about 20 Ad12 genome copies, as determined by the fluorescent *in-situ* hybridization (FISH) technique. (**b**) Schematic presentation of one of the Ad12 DNA junction sites with hamster cell DNA, as derived from the analysis of the nucleotide sequence at the site of linkage. The capital letters L, M, I and P refer to the *Pst*I fragments of Ad12 DNA, and 34 123 refers to the right-terminal nucleotide number in the Ad12 DNA sequence[8] in the terminal *Pst*I–L fragment of which two nucleotides are deleted (ΔL). T7 and SP6 are phage-polymerase sites in the vector into which the junction fragment has been cloned. Reproduced with permission from Ref. 8.

We conclude that the observed alterations in the IAP methylation patterns in λ-DNA-transgenic hamster cells are unlikely to preexist or to be caused by the transfection method. We favour the possibility that the insertion of foreign DNA into a preexisting mammalian genome affects the chromatin structure so that the patterns of DNA methylation are altered. The locations of the cellular sites altered by such changes may well depend on the site of foreign DNA insertion, but this possibility has not yet been investigated systematically.

We have initiated experiments to analyse changes in chromatin structure in transgenic cell lines. We have also started to investigate changes in DNA methylation patterns in mice that are transgenic for bacteriophage λ DNA (K. Müller and W. Doerfler, unpublished).

Uptake of foreign DNA via the gastrointestinal tract in mammals

The investigations of the fate of foreign DNA in mammalian cells propagated in culture have also been extended to similar analyses in living animals. These studies have been motivated by the question of what the target of this putative cellular defence mechanism

(*de novo* methylation of integrated foreign DNA) could in fact be in living organisms. The most obvious route for entry of foreign DNA in all mammals is the GI tract following ingestion with the food supply – the epithelium of the GI tract can be exposed to food-ingested DNA for many hours on a daily basis.

Using bacteriophage M13 DNA as a test molecule devoid of homology to mouse DNA, we have demonstrated that food-ingested foreign DNA is not completely degraded in the GI tract of mice[16]. The fate of this DNA was followed by dot-blot hybridization, the polymerase chain reaction (PCR) or FISH. In 84 animals, fragments of M13 DNA were detected in the contents of the small intestine, the caecum (up to 18 hours), the large intestine and in the faeces[3,16]. In 254 animals, M13 DNA fragments of up to 976 bp were found in the blood two to eight hours after feeding. In buffer-fed control animals, M13 DNA was not detected. M13 DNA fragments have also been traced by PCR in peripheral blood leukocytes and located by FISH in about 0.1% of the white blood cells between two and eight hours and in spleen or liver cells up to 24 hours after feeding. M13 DNA has, in addition, been observed in the nuclei of some of the columnar epithelial cells, in leukocytes in Peyer's patches of the caecum wall, in liver cells and in B cells, T cells and macrophages in the spleen by the use of FISH. These findings suggest transport of foreign DNA through the intestinal wall and Peyer's patches to peripheral blood leucocytes and into several organs. Figure 2 demonstrates the presence of GI-tract-ingested M13 DNA in the nuclei of cells in different organs, as detected by the FISH technique. However, M13 DNA has not yet been discovered by PCR or FISH in any organ system of the fed animals at times later than 24 hours after feeding.

Upon extended feeding, M13 DNA has been recloned from total spleen DNA into a λ vector. One of these clones was found to contain a ~1300 bp fragment of M13 DNA, as identified by DNA

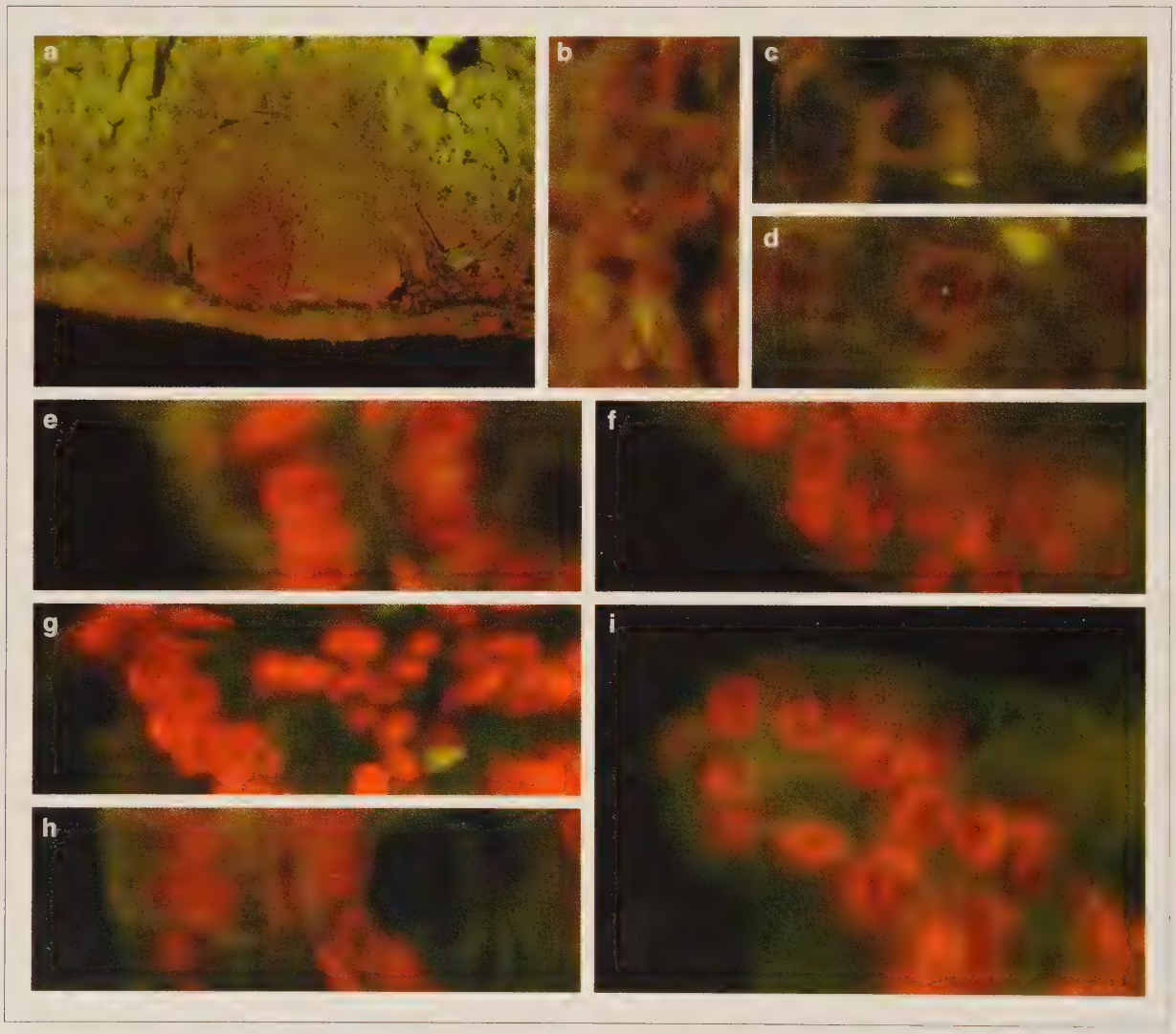

Figure 2

Persistence of food-ingested bacteriophage M13 DNA in the nuclei of cells from various organs of M13-fed and control mice. Experimental details are described in the text and in Refs 3 and 16. (**a**) Caecum wall, overview; (**b**) Peyer's patch in the caecum wall of an M13-fed animal; (**c**) unfed control, liver; (**d**) liver; (**e**)–(**i**) caecum. Data have been taken from Ref. 3.

sequencing. This M13 DNA fragment has been found to be covalently linked to an 80 bp DNA fragment with approximately 70% homology to the mouse IgE receptor gene[3] and to other known mouse DNA segments. These data indicate that the GI tract is apparently not a complete barrier to the uptake of fragments of food-ingested foreign DNA, but it is unknown how frequently this uptake will occur and what its potential consequences for the targeted host cell might be. In rare cases, this foreign DNA may become covalently linked to the cellular DNA, and the medical and evolutionary implications of this could be considerable.

Foreign DNA in mammalian organisms – possible implications for transgenic organisms and somatic gene therapy

Experimental schemes involving the integration of foreign DNA into established mammalian, and probably other eukaryotic, genomes have to take into account the possibility that these insertions may have far-reaching consequences, with unexpected alterations of methylation and expression patterns at chromosomal sites remote from the site of foreign DNA insertion. It will be the goal of this research to improve our understanding of the mechanisms and rules underlying these alterations in *trans* locations. It is important to emphasize that we have not found changes in all the clonal cell lines containing integrated λ DNA. Of course, it is possible that such changes may have occurred at sites different from the ones analysed, because we do not know whether changes in DNA methylation in cellular genes are elicited in every insertion event, or whether the foreign DNA has to be inserted at certain sites to lead to alterations of cellular DNA methylation. A number of interesting research issues have been raised for those interested in evaluating the interdependencies between chromatin structure, DNA methylation patterns and the consequences of foreign DNA integration into established genomes. The days of transgenic experimental accomplishments without considering the long-term consequences may fade rapidly into the past, and human somatic gene therapy, in particular, will have to consider carefully the long-term effects of foreign DNA insertion.

Acknowledgements

Research in the authors' laboratory was supported at different times by the Deutsche Forschungsgemeinschaft, grants SFB274-A1 and Do165/12-1; by the Wilhelm Sander-Stiftung, München, grant 94.004.1/2; by the Bundesministerium für Erziehung, Wissenschaft, Forschung und Technologie, Bonn – Verbundforschung, Az: BEO-0311110 and by the Fonds der Chemischen Industrie, Frankfurt.

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Severe defects in immunity and hematopoiesis caused by SHP-1 protein-tyrosine-phosphatase deficiency

Leonard D. Shultz, Thiruchandurai V. Rajan and Dale L. Greiner

Spontaneous mouse mutations that cause severe immunodeficiency or autoimmunity are invaluable tools with which to investigate the mammalian immune system. Mutations at the 'motheaten' locus result in severe immunological dysfunction due to disruption of the structural gene encoding Src-homology 2-domain phosphatase-1 (SHP-1). This natural model for a specific protein-tyrosine-phosphatase deficiency is being widely utilized to determine the role of SHP-1 in the negative regulation of multiple signaling pathways in a number of hematopoietic lineages.

More than 30 spontaneous single-gene mutations in the mouse cause marked defects in the development or regulation of the immune system[1,2]. Certain of these mutations are considered to be homologs for specific human diseases; for example, the beige mutation is a homolog of Chediak–Higashi syndrome, while the X-linked-immunodeficiency mutation is a homolog of X-linked agammaglobulinemia. Over the past few years, the molecular bases of ten immunological mutations have been elucidated[2]. The identification of these gene defects has increased our understanding of the development and regulation of the immune system and has led to the development of novel therapeutic agents designed to regulate the development and function of both normal and neoplastic immune cells.

The recent identification of the genetic basis for the motheaten and viable-motheaten mutations has been of considerable interest. The recessive motheaten and viable-motheaten allelic variants result in the most deleterious abnormalities in hematopoiesis and dysregulation of the immune system that are known to be caused by a single-gene mutation. The mutations are within the structural gene for a nontransmembrane protein-tyrosine phosphatase (PTP) expressed primarily in hematopoietic cells[3,4]. However, non-immunological defects, including infertility and decreased body weight, are also observed in motheaten and viable-motheaten mice. The PTP disrupted by these mutations had been cloned by several groups and given several names – PTP-1C, HCP, SHP, SHPTP1 and PTPN6 (reviewed in Ref. 3). Recently, it has been recommended that the phosphatase be renamed SHP-1 (Ref. 5), and this term will be used in this article.

In mice, the SHP-1 protein is encoded by a gene named hematopoietic cell phosphatase (*Hcph*), as defined by the *International Committee on Genetic Nomenclature for Mice*. The human locus has been named protein tyrosine phosphatase nonreceptor type 6 (*PTPN6*), as determined by the *Human Gene Mapping Nomenclature Committee*. The official gene symbols for the motheaten and viable-motheaten mutations are $Hcph^{me}$ and $Hcph^{me\text{-}v}$, respectively. For the purposes of this review, the abbreviated gene symbols *me* and me^{v} will be used.

The finding that the *me* and me^{v} mutations caused SHP-1 deficiency provided the first animal models of a specific PTP deficiency. These models have facilitated investigations of the role of SHP-1 in the development and regulation of the hematopoietic and immune systems in both the normal and the pathological states. This review will describe the phenotypic effects of the *me* and me^{v} mutations and provide an overview of recent studies on the determination of mechanisms by which SHP-1 deficiency causes the profound immunological and hematological

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PII: S0167-7799(97)01060-3

abnormalities in *me/me* and me^v/me^v mice. Identification of these molecular mechanisms should lead to the elucidation of novel signaling pathways and the determination of potential therapeutic targets for manipulation of hematopoietic cells in both normal and neoplastic conditions.

Immunological and hematological abnormalities in *me/me* and me^v/me^v mutant mice

The first reported mutation at the motheaten locus occurred in 1965 in the C57BL/6J mouse strain at The Jackson Laboratory[6]. Homozygotes (*me/me*) are recognizable at one to three days of age by the presence of neutrophilic aggregates in the epidermis. By two to three weeks of age, subepidermal accumulations of neutrophils displace hair follicles, which results in patchy absence of pigment. The skin abnormalities for which this mutant was first named occur even under specific pathogen-free husbandry conditions, indicating that infection does not underlie the gross phenotypic characteristics of *me/me* mice and is not responsible for their short lifespan. Impaired immunological function in *me/me* mice was evidenced by reduced proliferative responses to B- and T-cell mitogens, absence of cytotoxic-T-cell responses and severely reduced natural killer (NK)-cell function[1]. In addition to severe immunodeficiency, *me/me* mice develop systemic autoimmunity, which is characterised by polyclonal B-cell activation accompanied by hyperimmunoglobulinemia, expression of multiple autoantibodies and widespread inflammatory lesions, including severe pneumonitis[1]. The proximate cause of death of *me/me* mice appears to be pulmonary injury, characterised by interstitial pneumonitis accompanied by focal intra-alveolar hemorrhages with accumulations of alveolar macrophages and neutrophils in the lower respiratory tract[1]. The short lifespan of *me/me* mice constrained *in vivo* experimentation and so the basic defects in the immune system could not be distinguished from those associated with delayed maturation or with a moribund condition. The occurrence of the me^v mutation facilitated longer-term studies, because me^v/me^v mice have a three-fold increase in lifespan (nine weeks, compared with the three weeks of *me/me* mice)[7]. The increased lifespan was not associated with a marked improvement in immunological function but was rather correlated with a more chronic progression of the inflammatory lung disease. Table 1 summarizes the many immunological and hematological abnormalities found in *me/me* and me^v/me^v mice.

Genetic mapping of the motheaten locus and determination of the molecular basis for the genetic defect

In 1975, several linkage-testing stocks were used to map the *me* locus to the distal end of chromosome 6 (Ref. 6). More recently, high resolution mapping of this locus was carried out using an interspecific backcross between C57BL/6J–me^v/me^v and an inbred stock of *Mus musculus castaneus*. This mapping revealed that the *me* locus mapped to a region encompassing the hematopoietic cell phosphatase (*Hcph*) locus[3], which encodes the SHP-1 protein. Since SHP-1 is expressed primarily in hematopoietic cells and was thought to function as an important signaling molecule in hematopoietic cell growth and differentiation, the *Hcph* gene was considered to be an excellent candidate gene for the *me* mutation.

Abnormalities of SHP-1 in both *me/me* and me^v/me^v mice were demonstrated by Western blot analyses and by PTP activity assays. Molecular analyses of the *Hcph* DNA identified abnormal transcripts in both mutants. Both the *me* and me^v mutations were found to result in defective RNA splicing. As shown in Fig. 1, the *me* and me^v mutations were found to occur at different places in the gene. The *me* mutation is caused by the deletion of a cytidine residue, which generates a novel splice-donor site in the first Src-homology-2 (SH2) domain. This produces a frameshift and so causes premature truncation of the mRNA, resulting in a null mutation. This is corroborated by an absence of both *Hcph* mRNA and SHP-1 protein in *me/me* hematopoietic cells. In contrast, the me^v mutation is a thymidine-to-adenine transversion, resulting in the destruction of a splice-donor site. The activation of cryptic splice sites produces an in-frame insertion or deletion in the phosphatase catalytic domain. Abnormal SHP-1 protein in me^v/me^v hematopoietic cells retains approximately 20% of the wild-type activity[3,8].

Role of SHP-1 deficiency in immunopathological abnormalities

Since the discovery that the *me* and me^v mutations resulted either in an absence of or a severe reduction in SHP-1 activity, respectively, numerous investigations have focused on the mechanisms by which SHP-1 deficiency causes the myriad immunopathological changes found in mutant mice. The studies discussed below demonstrate that SHP-1 is a critical down-regulatory molecule in multiple signaling pathways used by lymphoid as well as myeloid cells.

Overgrowth of macrophages and granulocytes

A major immunopathological abnormality in *me/me* and me^v/me^v mice is the overgrowth of macrophages and granulocytes in the lungs, skin and extremities[6,7]. The accumulation of macrophages and neutrophils in the lungs results in a fatal pneumonitis, while inflammation in the extremities is associated with arthritic changes in the joints accompanied by autoamputations, particularly in the rear paws. The accumulation of myeloid cells in these tissues does not require the presence of T- or B-cells, because me^v/me^v mice rendered either T- or B-cell-deficient following genetic crosses with the $Hfh11^{nu}$ mutation[9] or the *Igh-6* targeted mutation (L. D. Shultz, unpublished data), respectively, still develop severe macrophage and granulocyte inflammatory lesions. Similarly, genetic crosses between either recombination-activating-gene-1 (*Rag1*)-knockout[10] or *scid/scid* mice (L. D. Shultz, unpublished data) and me^v/me^v mice have

Table 1. Immune-system and hematopoietic abnormalities in motheaten and viable motheaten mice[a]

Cell population	Phenotype
B cells	Polyclonal B-cell activation, CD5 expression, accumulation of plasmacytoid Mott cells
T cells	Dysfunctional, early thymic involution, increased thymocyte proliferation following T-cell-receptor stimulation
Macrophages	Accumulation in lungs and elsewhere
Neutrophils	Accumulation in skin, lungs and blood
Natural killer cells	Decreased activity
Langerhans cells	Marked depletion of epidermal dendritic cells
Red blood cells	Hemolytic anemia
Mast cells	Increased numbers of dermal mast cells
Bone marrow stromal cells	Failure to support hematopoiesis *in vitro*
Stem cells	Development of disease in bone-marrow chimeras

[a]Reviewed in Refs 1,3,24.

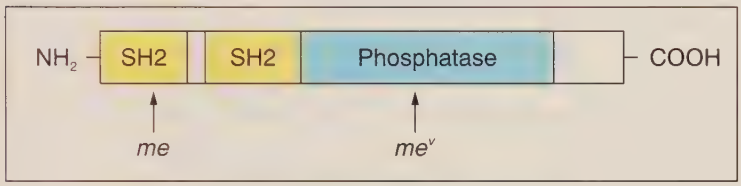

Figure 1

The coding region of the murine *Hcph* gene. Arrows indicate the location of the *me* and *me*^{v} mutations. The *me* mutation is caused by the deletion of a cytidine residue, generating a novel splice donor site and resulting in premature truncation. The *me*^{v} mutation is a thymidine-to-adenine transversion that destroys a splice donor site, resulting in activation of cryptic splice sites. In-frame insertion or deletion in the phosphatase catalytic domain results in the production of abnormal protein products.

confirmed that neither B- nor T-cells are required for the development of macrophage and granulocyte inflammatory lesions.

In vitro studies have shown that the accelerated growth of *me/me* and *me*^{v}/*me*^{v} macrophages and production of transforming growth factor-1 (TGFβ-1) are associated with suppression of lymphopoiesis and impaired development of NK cells[11–13]. Bone-marrow-progenitor cells from *me/me* and *me*^{v}/*me*^{v} mice show enhanced proliferative responses *in vitro* to macrophage-colony-stimulating factor (CSF-1)[14], granulocyte/macrophage-colony-stimulating factor (GM-CSF)[15] and granulocyte-colony-stimulating factor (G-CSF)[16]. These studies suggest that SHP-1 is a critical negative regulator of cytokine-receptor signaling in macrophages and granulocytes. Evidence supporting the negative regulatory function of SHP-1 in the cytokine-receptor signaling of monomyeloid cells has emerged from a number of studies.

(1) Tyrosine residues in the CSF-1 receptor are rapidly phosphorylated in response to CSF-1 stimulation, and the tyrosine kinase domain is activated[17]. In the absence of SHP-1, the CSF-1 receptor of *me/me* bone-marrow macrophages is hyperphosphorylated, and a number of associated proteins appear to be hyperactivated following CSF-1 stimulation, resulting in recruitment of SH2-domain-containing proteins, including PI-3 kinase and Grb-2 (Ref. 14). However, recent studies with *me*^{v}/*me*^{v} bone-marrow macrophages have not confirmed hyperphosphorylation of the CSF-1 receptor[18].

(2) Low levels of proliferation of *me/me* and *me*^{v}/*me*^{v} bone-marrow macrophages in culture occur in the absence of CSF-1[15].

(3) The increased proliferative response of *me*^{v}/*me*^{v} bone-marrow cells to GM-CSF is accompanied by the hyperphosphorylation of several proteins, including a novel 126 kDa phosphotyrosine protein that associates with SHP-1 via its SH2 domain, suggesting that these proteins depend on SHP-1 for dephosphorylation and that SHP-1 deficiency may mediate the heightened response of *me/me* and *me*^{v}/*me*^{v} progenitor cells to GM-CSF[15].

(4) Enhanced proliferative and colony-forming responses of *me*^{v}/*me*^{v} bone marrow cells to G-CSF are associated with increased numbers of immature granulocytes expressing the G-CSF receptor[16]. Although SHP-1 does not appear to associate directly with the G-CSF receptor, it associates with an unidentified 92 kDa phosphoprotein[16].

(5) The receptor for GM-CSF lacks intrinsic kinase activity, and includes a unique alpha subunit, which binds ligand, and a common beta subunit. This latter subunit is also utilized by the interleukin (IL)-3 and -5 receptors and is responsible for receptor signaling following cytokine binding[19]. It has been shown that SHP-1 associates with this beta chain, resulting in the downregulation of IL-3 signaling[20]. The important role of monomyeloid cells in mediated pathological changes in *me/me* and *me*^{v}/*me*^{v} mice *in vivo* is shown by the ability of anti-Mac-1-antibody treatment to inhibit inflammatory disease *in vivo*[21].

It has been speculated that circulating immune complexes in *me/me* mice might react with FcγR1 receptors on macrophages and neutrophils, contributing to the abnormal activation of these cells[22]. Interferon (IFN)-α/β appears to be involved in increased FcγR1 expression. Two recent investigations have shown that SHP-1 plays an important role in the regulation of IFN-α/β signaling[22,23]. SHP-1 is a negative regulator of IFN-α/β signaling through an ability to associate physically with the IFN-α/β-receptor signaling complex. In the absence of SHP-1, IFN-α/β signaling through Janus kinase 1 (Jak1) and Stat-1α tyrosine phosphorylation is greatly increased[22]. These studies, when taken together, suggest that many cytokine-receptor signaling pathways in monomyeloid cells are activated by tyrosine phosphorylation, and that dephosphorylation by SHP-1 mediates a regulatory signal to deactivate these signaling pathways.

Abnormal B-cell development and polyclonal B-cell activation

Many signaling pathways in lymphocyte development, activation and apoptosis involve protein-tyrosine phosphorylation. As a result, the nature of the lymphoid

abnormalities in *me/me* and *me^v/me^v* mice has been under intense study. Defects in B-cell development in *me/me* and *me^v/me^v* mice include a severe reduction in numbers of early B-cell progenitors in the bone marrow accompanied by a polyclonal B-cell activation and accumulations of large numbers of abnormal plasmacytoid cells termed Mott cells[1,24]. Although *me/me* and *me^v/me^v* mice have reduced numbers of splenic B cells, the majority of the remaining B cells present express the pan-T-cell glycoprotein Ly-1 (CD5). CD5$^+$ B cells normally constitute a large percentage of adult peritoneal and newborn splenic B-cell populations, but are a rare population in the spleens of normal adult mice[25]. Genetic crosses between *me/me* mice and X-linked-immunodeficiency (*Btk^{xid}*)-mutant mice, which are deficient in Bruton's agammaglobulinemia tyrosine kinase[26], causes the expansion of the CD5$^+$ B-cell population to be blocked, reducing the polyclonal B-cell activation without increasing the lifespan of the *me/me– Btk^{xid}/Y* mice beyond that of *me/me* mice[27]. B cells from *me^v/me^v* mice and CD5$^+$ B cells from normal mice show enhanced proliferation following exposure to phorbol myristate 13-acetate, a protein-kinase-C agonist that induces tyrosine phosphorylation in these cells[28]. This suggests that CD5$^+$ B cells in normal mice may express decreased levels of SHP-1 activity and fail to downregulate a signaling pathway important in CD5$^+$ B-cell activation.

The molecular mechanism by which SHP-1 deficiency results in polyclonal B-cell activation is under study in a number of laboratories. In normal B cells, antigen-receptor signaling involves the activation of several nontransmembrane protein tyrosine kinases (PTKs), including Syk and Lyn, followed by phosphorylation and the recruitment of downstream signaling molecules[29]. However, exposure of normal B cells to antigen in the form of immune complex inhibits membrane-immunoglobulin (mIg)-mediated signaling. This inhibition of B-cell activation results from the recognition of the IgG-constant-region Fc domains by the FcγRIIB1 receptor[30]. Phosphorylation of the FcγRIIB1 receptor in normal B cells leads to binding of the SHP-1 carboxy-terminal SH2 domain, accompanied by a marked increase in SHP-1 activity and decrease in B-cell signaling[31]. In *me^v/me^v* B cells, SHP-1 deficiency results in a failure of inhibitory signaling following coligation of the FcγRIIB1 receptor[31,32]. This leads to increased B-cell-receptor (BCR) signaling and polyclonal B-cell activation. The deficiency of SHP-1 in *me^v/me^v* mice allows a lower threshold of signaling for negative selection, lower valency requirements for specific antigen binding and increased elimination of developing B cells[33].

BCR signaling and negative selection are also regulated by CD22, a cell-surface phosphoglycoprotein. CD22 tyrosine residues are rapidly phosphorylated following BCR crosslinking, promoting association between CD22 and SHP-1[34]. Tyrosyl-phosphorylated CD22 binds to the amino-terminal SH2 domain of SHP-1, and dephosphorylation of CD22 results in decreased BCR signaling and negative regulation of B-cell function[35,36]. Evidence for the role of the CD22–SHP-1 signaling pathway as a negative regulator of BCR signaling includes recent analyses of mice whose CD22 was disrupted by homologous recombination. Targeted disruption of the CD22 structural gene resulted in B-cell abnormalities similar to those observed in *me/me* and *me^v/me^v* mice, supporting the hypothesis that the lack of appropriate CD22 dephosphorylation by SHP-1 underlies certain of the B-cell abnormalities in these mice[37]. B-cell abnormalities in CD22-null mice include hyperresponsiveness to BCR signaling accompanied by increased IgM levels, production of autoantibodies and expansion of the peritoneal CD5$^+$ B-cell population[37]. SHP-1 is also constitutively associated with a signaling molecule termed the SH2-containing leukocyte-specific protein of 76 kD (SLP-76)[38]. Although the functional relevance of SHP-1 and SLP-76 interaction is not yet well understood, it is becoming increasingly clear that the SHP-1 protein regulates B-cell signaling pathways by interaction with a number of other signaling regulatory proteins.

T-cell developmental abnormalities and impaired Fas-mediated apoptosis

Although the thymuses of *me^v/me^v* mice appear histologically normal at one to three weeks of age, thymic involution begins by three to four weeks of age[7]. This early thymic involution is associated with an accessory-cell defect. Bone-marrow cells from these mice are unable to repopulate the thymus of lethally irradiated recipients following intravenous injection, but those from both *me/me* and *me^v/me^v* mice can generate normal numbers of thymocytes following direct intrathymic injection[39]. Subsequent experimentation revealed that the early thymic involution was associated with defective homing of a putative intrathymic-accessory-cell precursor[40,41]. These studies suggest that the SHP-1 defect may be expressed in a nonlymphoid hematopoietic progenitor cell.

Defects in responses to activation signals have been described in *me/me* and *me^v/me^v* thymocytes. Several nontransmembrane PTKs, including Lck and Fyn, are associated with the T-cell receptor (TCR) and are involved in early thymocyte maturation[42]. The finding that SHP-1 undergoes Lck-dependent tyrosyl phosphorylation in both primary normal thymocytes and in a T-cell-hybridoma cell line indicated that SHP-1 may function in the regulation of early T-cell-signaling events[43]. If SHP-1 functions as a negative regulator of TCR signaling, it would be expected that the lack of SHP-1 in *me/me* thymocytes would result in increased Lck and Fyn activation in response to TCR or CD4 stimulation. This expectation was confirmed by the demonstration that increased activation of Lck and Fyn in *me/me* thymocytes was accompanied by increased tyrosyl phosphorylation of several intracellular substrates[44]. The functional consequence of the impaired negative regulation was increased

proliferative activity of *me/me* thymocytes in response to TCR stimulation[43]. SHP-1 was recently shown to regulate T-cell activation by its association with the protein-tyrosine kinase ZAP-70. This interaction results in an increase in phosphatase, and a concomitant decrease in kinase, activity, thus regulating TCR signaling[45]. The inhibitory effect of SHP-1 on TCR signaling appears to be mediated by events occurring at the level of the TCR complex[46] as well as at the level of downstream signaling elements that couple the activated TCR to the Ras–MAPK (mitogen-associated kinase) response pathway[47]. The precise biological relationship between the lack of inhibitory signals in thymocytes, the expression of functional T-cell defects and the presence of autoimmunity in *me/me* and me^v/me^v mice remains to be determined.

Although there is clearly not an obligate role for T cells in the development of pneumonitis or skin lesions, the increased proliferation of $CD3^+$ T cells in the lungs of me^v/me^v mice indicates that T cells are recruited to these inflammatory sites. The presence of T cells in inflammatory lesions appears to be associated with a defect in apoptosis. Fas (APO-1 or CD95) is a critical mediator of programmed cell death in activated T cells[48]. It has been suggested that SHP-1 is required for Fas-mediated apoptosis, because me^v/me^v mice show decreased apoptosis following exposure to anti-Fas antibody despite having increased Fas expression in lymphoid tissues[49]. Subsequent studies showed that me^v/me^v mice show prolonged T-cell proliferation following anti-CD3 stimulation associated with defective Fas-mediated apoptosis of activated T cells[50]. However, the role of SHP-1 as a critical regulator of Fas-mediated apoptosis has not been confirmed[44,51] and awaits further clarification.

Association of SHP-1 with NK-cell inhibitory receptors

Inhibitory receptors on NK cells recognise 'self' major histocompatibility complex (MHC) class I expression on potential target cells. This recognition by the killer-inhibitory receptors (KIR) mediates resistance to NK cell lysis. Recognition of the HLA-C major histocompatibility antigen by human NK cells is mediated by an immunoglobulin-related molecule termed p58. The inhibitory function of the p58 KIR has been shown by several groups to involve tyrosine phosphorylation of the receptor and recruitment of SHP-1.

KIRs on mouse NK cells are characterized by extracellular lectin domains. One of these KIRs, the Ly-49A receptor, recruits SHP-1, resulting in inhibitory activity[52]. Ly-49A associates directly with SHP-1, interrupting proximal signaling events critical to the cytotoxic function of NK cells[53]. SHP-1 is known to be required for optimal function of Ly-49A because such function is impaired in NK cells isolated from SHP-1-deficient *me/me* mice[53]. Thus, it appears that SHP-1 delivers a negative signal that interferes with target-cell lysis by NK cells in both mouse and man. The paucity of NK-cell activity in *me/me* mice may be related to the loss of such cells as a consequence of the unregulated lysis of 'self' hematopoietic-cell populations or the exhaustion of NK cells through continuous stimulation.

Abnormalities in red-blood-cell and mast-cell development

Abnormal erythroid-lineage development in me^v/me^v mice is characterised by a 90-fold increase in the frequency of erythroid precursors (CFU-E) in the spleen[54]. These CFU-E show increased sensitivity to erythropoietin (EPO) and include a population of EPO-independent red-blood-cell (RBC) progenitors. The role of SHP-1 as a negative regulator of erythropoiesis has recently been elucidated[55]. Following binding of EPO to its receptor, the protein-tyrosine kinase Jak2 is phosphorylated and binds to the inner portion of the receptor. Concomitant phosphorylation of the EPO receptor creates docking sites for other downstream signaling molecules. The capacity of Jak2 to autophosphorylate is inhibited by SHP-1, which binds to the EPO receptor by its SH2 domains[55]. Recently, it has been shown that SHP-1 can bind directly to and dephosphorylate Jak2 in an SH2-independent manner[56]. The lack of SHP-1 activity in *me/me* and me^v/me^v mice leads to continuous activation of the EPO signaling pathway and expansion of erythroid precursors. Although me^v/me^v mice have greatly increased numbers of splenic CFU-E accompanied by a four-fold increase in the numbers of reticulocytes in the blood, these mice develop macrocytic anemia[7,54]. A simple interpretation of these observations would suggest a shortened lifespan for erythrocytes in the circulation. The known function of SHP-1 in erythropoiesis would predict that SHP-1-deficient mice would develop elevated RBC levels. These symptoms have, in fact, been reported, in a Finnish family whose members have a mutation in the EPO-receptor gene that prevents expression of the SHP-1 docking site on the EPO receptor[57]. The anemia in me^v/me^v mice is therefore most likely to be due to the elimination of circulating RBC by autoantibodies and/or activated macrophages.

Mast-cell development in *me/me* and me^v/me^v mice is also abnormal, with elevated numbers of mast cells apparent in the skin. Mice carrying mutations at the dominant spotting (Kit^w) locus, which ecodes the c-Kit receptor, have pleiotropic defects including severe depletion of mast-cell populations and develop macrocytic anemia. SHP-1 associates transiently with c-Kit following ligand stimulation[58]. Genetic crosses between *me/me* mice and mice carrying mutant alleles at the Kit^w locus reveal that homozygosity for both mutations results in a decreased severity of lung inflammation and normalization of mast cell numbers, without affecting the anemia[59,60].

Conclusions

In summary, mutations at the *Hcph* locus, encoding the SHP-1 protein-tyrosine phosphatase, cause pleiotropic effects in multiple cell lineages including macrophages, granulocytes, lymphocytes, NK cells,

erythrocytes and mast cells. Given the central role of SHP-1 as a negative regulator of signal transduction, the pleiotropic effects of the *me* and *me^v* mutations are not surprising. While abnormalities inherent in individual cell lineages are responsible for certain hematopoietic and immunological defects in these mutant mice, many of the defects are likely to be associated with abnormalities in the interaction between lymphoid and myeloid cell lineages. Although there are still difficulties in determining the precise mechanisms by which SHP-1 deficiency results in the myriad pathological changes in *me/me* and *me^v/me^v* mice, rapid advances in the understanding of the role of SHP-1 in signal transduction promise further progress. Future studies of *me/me* and *me^v/me^v* mice will expand our knowledge of the role of the SHP-1 protein in the regulation of the immune system in both the normal and the pathological states.

Acknowledgements

Research in the authors' laboratories was supported by National Institutes of Health grants CA20408 (LDS), AI30046 (TVR) and DK36024 (DLG). We thank James Matthews, Peter Schweitzer and David Serreze for critical reading of this review.

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Flux analysis of underdetermined metabolic networks: the quest for the missing constraints

Hendrik P. J. Bonarius, Georg Schmid and Johannes Tramper

Traditionally, the intracellular fluxes of complex metabolic networks were quantified by isotopic-tracer experiments, but, owing to practical limitations, 'metabolic-flux balancing' is emerging as an alternative. This has become an important tool for the quantitative analysis of the physiology of microorganisms and mammalian cells. It has been successfully applied to finding potential sites for metabolic engineering, determining metabolic capabilities and designing optimal feeding strategies. However, it has the fundamental problem that metabolic networks, and cyclic metabolic pathways in particular, are underdetermined. The search for constraints that can be used to determine fluxes correctly for a range of different conditions is an exciting challenge.

Microorganisms are widely exploited for the synthesis of proteins or low molecular weight compounds and to transform substrates into a wide variety of products. As with chemical-production plants, the flux distribution determines the efficiency of the production process. In biological systems, nutrients are only partly used for biomass and product synthesis, the remainder being used for energy supply. In addition, waste products accumulate and significant energy dissipates in futile cycles. In order to optimize the capacity of microbial metabolism, and thus increase yields, the intracellular metabolite flows have to be quantitated.

Traditionally, metabolic fluxes have been determined by isotopic-tracer experiments. As the carbon stoichiometry of metabolic reactions is known, it is possible to determine the fluxes at certain branch points by tracing the metabolic fate of carbon-labelled substrates. Although these isotopic-tracer techniques are well established and have been significantly improved, particularly by the application of nuclear magnetic resonance (NMR) technology to biological systems[1], they are laborious and expensive to conduct and cannot be used on an industrial scale. As an alternative, 'metabolic-flux balancing'[2], which requires the measurement of only the extracellular metabolites (Box 1), has been proposed as a means of determining the flow through the primary metabolic pathways[3].

Data from isotopic-tracer experiments have been supplemented with mass-balance equations of the relevant metabolites for the determination of fluxes in entire metabolic networks[4–7]. Additionally, computational methods have been developed to combine these different types of information, allowing the calculation of metabolic fluxes with a minimum of algebraic manipulation[5,6]. However, for industrial applications, it is desirable to determine intracellular metabolic fluxes by metabolic-flux balancing alone, and, because no isotopically labelled substrate is required, the estimation of fluxes from mass balances (see Glossary) can be made in virtually any biological or reactor system regardless of scale.

On-line applications of 'crude' stoichiometric analysis have already been shown to be effective as a means of improving production yields in fermentation processes[8]. It is theoretically possible to measure and control metabolic conversions on the level of single enzymatic reactions by metabolic-flux-balancing techniques, with the measurement of only a limited number of extracellular metabolites[9]. Apart from controlling complex metabolic networks or tracing potential sites for metabolic engineering, other applications (such as rational medium design[10], the elucidation of metabolic and toxicological effects, location of metabolic control[11], determination of maximum

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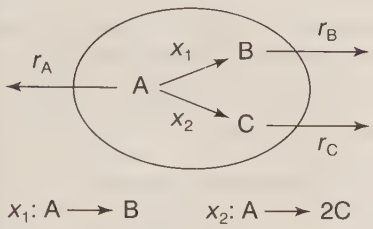
PII: S0167-7799(97)01067-6

Box 1. Principles of metabolic-flux balancing



Mass balances

over A: $r_A = -3 = -x_1 - x_2$

over B: $r_B = 1 = x_1$

over C: $r_C = 4 = 2x_2$

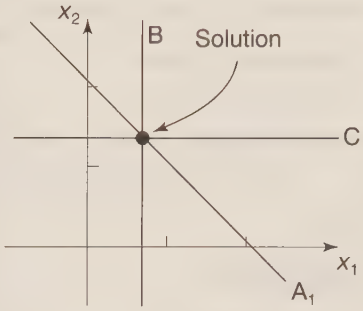


Figure. Principles of metabolic-flux balancing. (Numerical values are chosen arbitrarily.)

Metabolic-flux-balancing techniques are based on relatively simple linear algebra. If the stoichiometry of the relevant intracellular reactions and the cellular composition are known, and the uptake and secretion rates of the relevant metabolites (for example r_A, r_B and r_C in the Fig.) have been measured, the reaction rates (x_1 and x_2 in the Fig.) can be determined using the appropriate mass-balance equations. A reaction network is shown for which one unique solution for the variables x_1 and x_2 can be estimated by least-squares analysis of mass balances A, B and C. The least-squares method, which is used here because there are more constraints (mass balances) than unknowns (fluxes), is calculated by (pseudo)inverting stoichiometric matrix **A** (Ref. 3):

$$\mathbf{A}x = r \Leftrightarrow \mathbf{A}^T\mathbf{A}x = \mathbf{A}^T r \Leftrightarrow x = (\mathbf{A}^T\mathbf{A})^{-1}\,\mathbf{A}^T r$$

For the stoichiometry and measured metabolic rates given in the figure, this equation reads:

$$\begin{bmatrix} -1 & -1 \\ 1 & 0 \\ 0 & 2 \end{bmatrix} \cdot \begin{bmatrix} x_1 \\ x_2 \end{bmatrix} = \begin{bmatrix} r_A \\ r_B \\ r_C \end{bmatrix} \Leftrightarrow \begin{bmatrix} x_1 \\ x_2 \end{bmatrix} = \frac{1}{9}\begin{bmatrix} 5 & -1 \\ -1 & 2 \end{bmatrix} \cdot \begin{bmatrix} -1 & 1 & 0 \\ -1 & 0 & 2 \end{bmatrix} \cdot \begin{bmatrix} -3 \\ 1 \\ 4 \end{bmatrix} = \begin{bmatrix} 1 \\ 2 \end{bmatrix}$$

This shows that intracellular fluxes can be quantified by measuring only the uptake and secretion rates of the relevant metabolites.

theoretical yields[12] and quantitative prediction of biochemical phenotypes from gene data banks[13]) will all benefit from this computational technique. In this article, the fundamental problem that currently hinders the development of such a method is discussed and various proposed solutions are reviewed. Finally, experiments are suggested to investigate which of these solutions might lead to a method for quantifying metabolic fluxes that uses only mass balances.

Problem definition

Two fundamental problems have been identified in applying this technique to large, complex metabolic networks[3]. First, the estimated flux vector, which is calculated by the least-squares method, may be sensitive to slight perturbations in the measured extracellular rates of the relevant metabolites (r_A, r_B and r_C in Box 1). This sensitivity to error propagation can be checked by calculating the condition number of the system[3,14] (see Glossary), which depends solely on the reaction stoichiometry of the metabolic network. The condition number can be seen to increase with the complexity (Fig. 1a) and the ratio (Fig. 1b) of the stoichiometric coefficients. A large condition number (>100) indicates that the estimated flux distribution is sensitive to measurement errors. Combinations of reactions that cause network sensitivities can be identified using an algorithm developed by Savinell and Palsson[14]. The flow through these reactions either has to be determined independently by isotopic-tracer experiments or removed from the network. However, with a few exceptions[15], the condition number of a metabolic network is generally small (<100), which indicates that error propagation is insignificant[3]. Nevertheless, the condition number of the stoichiometric matrix should be determined prior to least-squares analysis.

A special case of error-sensitive metabolic networks is reaction dependency (Fig. 1c). In contrast to error propagation, this causes observability problems in the determination of the fluxes in the metabolic networks of almost all prokaryotic[9,12,15–18] and eukaryotic[15,19,20] cells. Reaction dependency occurs particularly in cyclic pathways, which are abundantly present in metabolic systems. In Fig. 2a, the problem of a cyclic metabolic pathway is visualized. Reactions in metabolic cycles are linear dependent (see Glossary) and cannot be derived from the extracellular rates. In a metabolic network, such reactions cause singularities, as a result of which the set of mass-balance equations is underdetermined. It is also apparent from Fig. 2 that the mass-balance equations of metabolites A and B yield the same information, as a result of which the fluxes x_1 and x_2 cannot be quantitated solely by

Glossary

Metabolite (or 'mass') balance	An equation that describes the accumulation and all relevant incoming and outgoing fluxes of a metabolite pool.
Stoichiometric matrix	A matrix that contains information on the reaction stoichiometry of cellular metabolism. The rows and columns of the stoichiometric matrix are associated with the **metabolite balances** and the metabolic fluxes, respectively.
Linear dependency	Metabolite balances are linear dependent if (a linear combination of) the solution planes determined by the **metabolite balances** are parallel. (For example, the mass balances of A and B in Fig. 2 are linear dependent.)
Rank	The maximum number of **linear-independent metabolite balances** in a metabolic network is called the rank of the **stoichiometric matrix**.
Rank deficient	If the **rank** is smaller than the number of metabolic fluxes (the number of rows of the **stoichiometric matrix**), then the metabolic network is rank deficient.
Condition number	The condition number of a **stoichiometric matrix A** (the ratio of the largest to smallest eigenvalue of **A**) is a measure of the sensitivity of the equation $\mathbf{A}x = r$ (Box 1).
Underdetermined networks	Metabolic networks that are **rank deficient** are designated 'underdetermined' to indicate that there are insufficient **linear-independent metabolite balances** to determine the intracellular metabolic fluxes.
Observability	In this context, the extent to which intracellular metabolic fluxes can be determined by the measurement of the extracellular metabolic rates and the biomass composition.
Directionality constraint	The demand that a (number of) flux(es) is non-negative.
Balanceable metabolite	A metabolite whose **mass balance** can be closed.

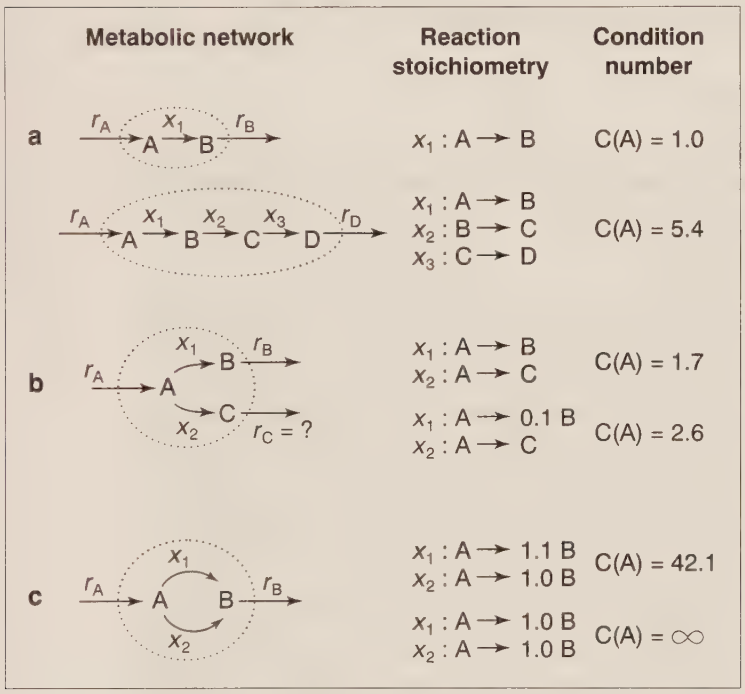


Figure 1

The error sensitivity of a metabolic network is dependent only on the reaction stoichiometry and can be determined by the condition number of the stoichiometric matrix[3]. (**a**) The condition number increases with growing complexity. (**b**) If only r_A and r_B are known, the fluxes x_1 and x_2 can be estimated. It is shown that the condition number increases with the ratio of the stoichiometric coefficients. (**c**) Networks that contain cyclic pathways are error sensitive or not observable [$C(A) = \infty$].

flux-balancing techniques. In this case, the solution space that contains all admissible solutions for fluxes x_1 and x_2 can be visualized by a single line (Fig. 2a). Underdetermined networks such as that shown in Fig. 2a are rank deficient (see Glossary).

Theoretical and experimental solutions

Over the past few years, various approaches have been proposed for the estimation of the fluxes of large, complex metabolic networks by supplementing the underdetermined network with various theoretical assumptions or constraints[3,9,12,15,18–21]. This is particularly important for the determination of fluxes in major metabolic cycles such as the TCA (tricarboxylic acid) cycle, the pentose-phosphate cycle and the malate shunt (the quantification of fluxes in 'futile cycles', where there is no branching of the cycle[22], is not covered in this review). Currently, several research groups are comparing flux distributions estimated on the basis of such assumptions with experimentally determined fluxes using isotopic-tracer methods (Ref. 7; K. Schmidt *et al.*, unpublished). Here, several 'candidate' constraints are reviewed and ways are suggested to find a method for the determination of intracellular fluxes without using isotopic tracers.

Mass balances of cofactors or co-metabolites as additional constraints

When a co-metabolite is produced or consumed in cyclic pathway reactions, the addition of its mass balance may yield a unique solution. In Fig. 2b, it is shown that a least-squares solution exists for a cyclic pathway when the mass balance of metabolite C is added to the network. In reality, addition of the mass balance(s) of co-metabolites is generally not sufficient to generate an (over)determined system. In complex networks, co-metabolites are either produced in more than one cyclic pathway (e.g. carbon dioxide) or are not balanceable (e.g. ATP). In most organisms, carbon dioxide is produced in the pentose cycle, the TCA cycle and the malate shunt and consumed in the pyruvate carboxylase reaction (Fig. 3). Therefore, the

addition of the carbon dioxide balance to the metabolic network will not allow an independent determination of the fluxes in these cycles. Nevertheless, useful information can be obtained from the carbon dioxide production rate, as it provides a means of checking the consistency of the estimated fluxes with respect to the carbon balance.

By addition of the ATP balance to a metabolite network, as shown in Fig. 3, the rank of the stoichiometric matrix increases by one unit, because the ATP balance is linear independent of the other mass balances. As a result, the observability increases and the split ratio of fluxes at either the glucose-6-phosphate or the pyruvate branchpoint can be determined. However, the ATP mass balance cannot be closed, owing to the fact that both ATP yields and ATP requirements for maintenance processes can only be estimated[9,15]. It has been suggested that theoretical calculations of ATP yields result in significantly higher levels than can be deduced from experimental data[23]. Moreover, relatively small changes in such estimates will have large effects on the calculated flux distribution.

The mass balances of reducing equivalents, for example NADH and NADPH, are often used to determine the split ratio of metabolic fluxes at branch points, such as occur in the metabolism at (phosphoenol)pyruvate or glucose-6-phosphate[9,15,17,20]. However, microorganisms interconvert these cofactors with unknown reaction rates using transhydrogenases. Therefore, in order to estimate fluxes in underdetermined networks, NADH and NADPH should be used as a lumped factor to circumvent the problem of transhydrogenase activity[20]. As a consequence, the split ratio of only one branch point (instead of two) can be estimated. Additionally, it has been shown for adipose tissue[19], hybridomas[7] and *Bacillus subtilis*[18] that the estimated flux distribution is very sensitive to the assumptions made or to small changes in the NAD(P)H balance.

Irreversibility of reactions as additional constraints

Some reactions in metabolic networks are considered irreversible. This additional information allows one to set lower boundaries to these particular reactions and to further constrain the solution space, in which all admissible solutions are situated. Although these constraints do not help to overcome observability problems, they can be used to fine tune the unconstrained solution to the equation in Box 1 (Ref. 3), if certain fluxes are negative where they should be irreversible.

An algorithm for the determination of all nonnegative, admissible fluxes has been described[24]. This computational method has been applied to describe the basic reaction modes, at the corner points of admissible solution space, in cyclic pathways[25] and to determine optimal flux distributions for the conversion of sugars to aromatic metabolites in *Escherichia coli*[26]. Furthermore, directionality constraints are essential when applying linear optimization techniques, as shown below.

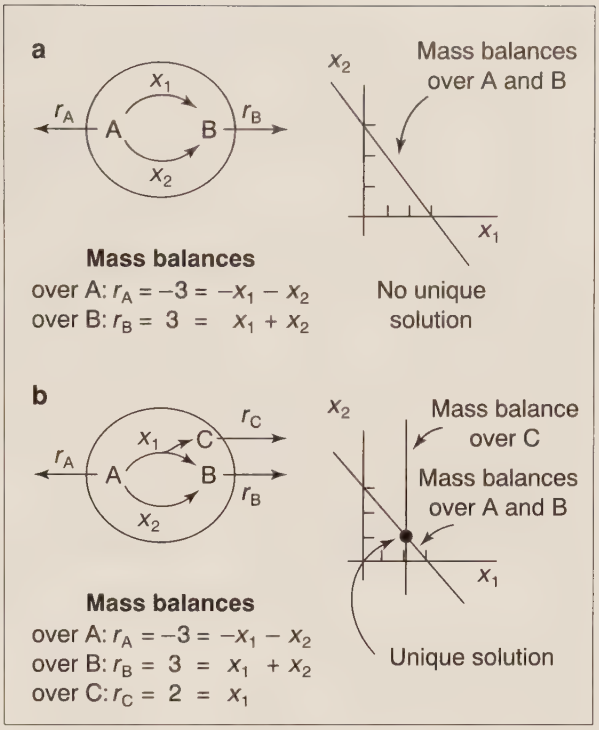


Figure 2
An undetermined (**a**) and a determined (**b**) metabolic network. (**a**) Reactions x_1 and x_2 are linear dependent. (Numerical values are chosen arbitrarily; all reactions are reversible.)

Objective functions as additional constraints; linear optimization

As pointed out above, the mass balances of cofactors such as ATP and NADH may be closed by mere approximation or remain unclosed, as certain biochemical parameters are not quantifiable. Instead, these mass balances can be used to formulate objective functions. Intracellular pools of cofactors for the transfer of energy or reducing power regulate many enzymatic reactions. Therefore a surplus or a lack of cofactors influences the flux distribution of entire metabolic networks. The metabolic pressure from such needs may be translated into linear objective functions and help to estimate optimal solutions that satisfy various metabolic goals[19]. Objective functions that have been applied to estimate fluxes in cyclic routes include, for example, 'maximize NADPH production'[19], 'minimize ATP production'[15] or 'minimize the sum of the squares of fluxes'[20].

Optimal solutions to all admissible flux distributions can be calculated using linear-optimization techniques. The principle of applying these techniques to estimate fluxes in underdetermined metabolic networks is shown in Fig. 4. By definition, linear optimization results in extreme ('optimal') solutions that are the end points of the stoichiometrically feasible domain[2]. In some cases, certain objective functions have no feasible solutions owing to the lack of lower or upper boundaries of cyclic routes. In Fig. 4 for example, this would be the case if the objective function were 'maximize NADH' and if the flux x_1 were *not* constrained instead of being irreversible. Lower

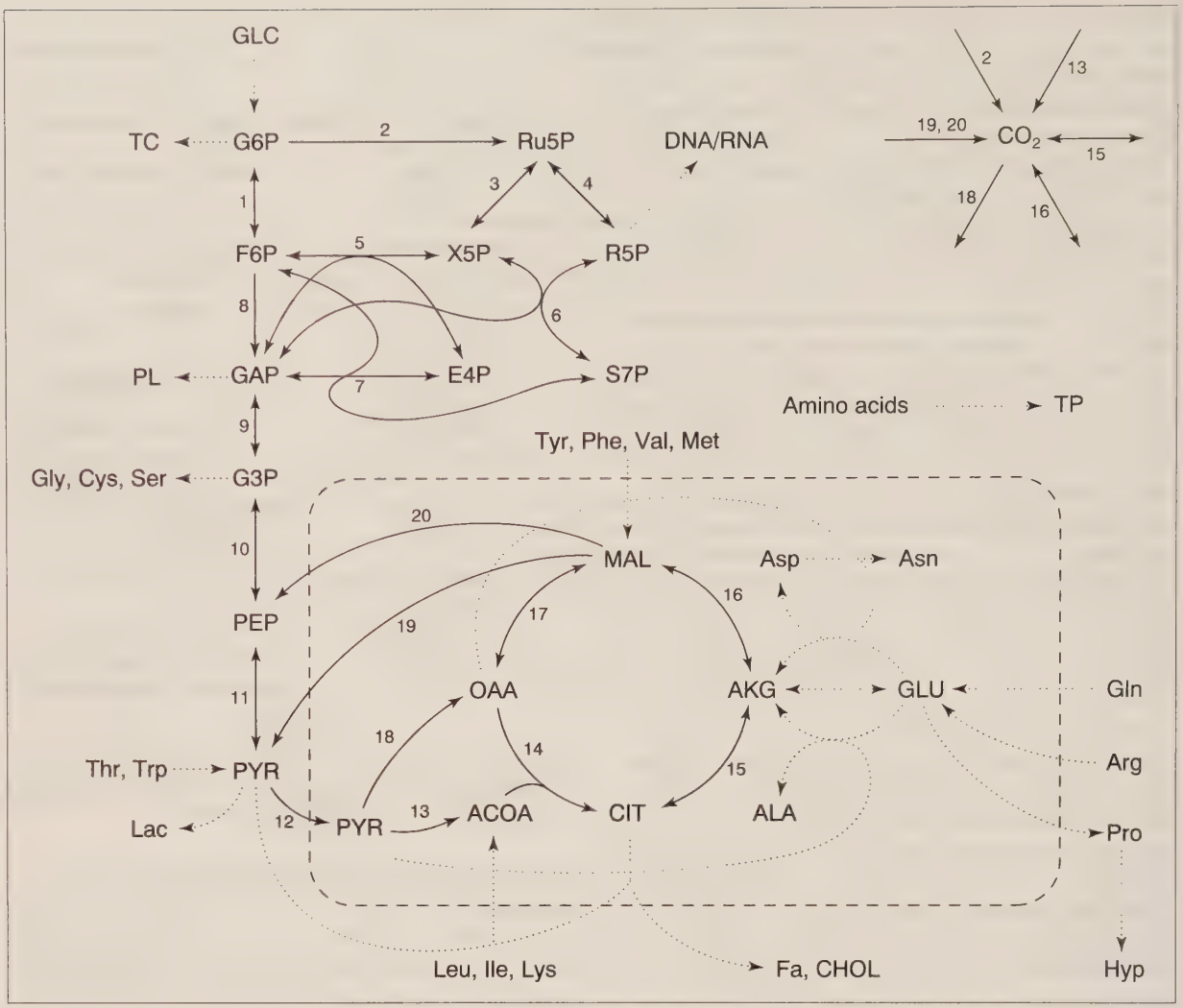


Figure 3

Some metabolic pathways of hybridoma cells[20]. Dashed arrows indicate fluxes that can be quantified using mass balances; solid, numbered arrows represent fluxes that are linear dependent and consequently not calculable using balancing techniques; the dashed line represents the mitochondrial membrane. Abbreviations: ACOA, acetyl-CoA; AKG, α-ketoglutarate; CHOL, cholesterol; CIT, citrate; E4P, erythrose-4-phosphate; GAP, glyceraldehyde-3-phosphate; GLC, glucose; G6P, glucose-6-phosphate; G3P, 3-phosphoglycerate; LAC, lactate; MAL, malate; OAA, oxaloactetate; PEP, phosphoenolpyruvate; PYR, pyruvate; R5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate; S7P, sedoheptulose-7-phosphate; TC, total carbohydrates; TP, total protein; X5P, xylulose-5-phosphate.

boundaries (which are determined by the irreversibility of fluxes) are essential for obtaining a feasible solution by linear optimization, especially because data for maximum reaction rates are not usually available. However, it is unclear whether the end points of the solution space that is constrained by the mass-balance equations represent the true flux distributions. The answer to this question can be found by isotopic-tracer experiments.

Finding the missing constraint

Little research has been carried out to validate the theoretical assumptions required to determine fluxes in rank-deficient networks by isotopic-tracer methods. It is therefore desirable to compare the flux distributions estimated on the basis of mass balances with the flux distributions determined by labelling experiments for a large range of growth conditions. In this way, a fundamental understanding of the effects of different constraints may be obtained, and it may be determined whether certain theoretical assumptions are generally valid. There are various methods for inducing drastic changes in the primary metabolism of microorganisms. A limitation of important carbon or nitrogen sources or electron acceptors (e.g. oxygen) will force microorganisms to rearrange their flux distribution. Alternatively, an increase in the growth rate, which can be induced by raising the dilution rate of cells in continuous culture, would result in an elevated NADPH requirement for anabolic processes. Theoretically, this will result in higher activity of two cyclic pathways, the pentose-phosphate shunt and the malate shunt. Another possible method for specifically stimulating certain fluxes is the addition of sublethal concentrations of toxic compounds with known effects on metabolism. For example, phenazine methosulphate specifically reoxidizes NADH and NADPH[27], resulting in an increase in NAD(P)H-producing reactions.

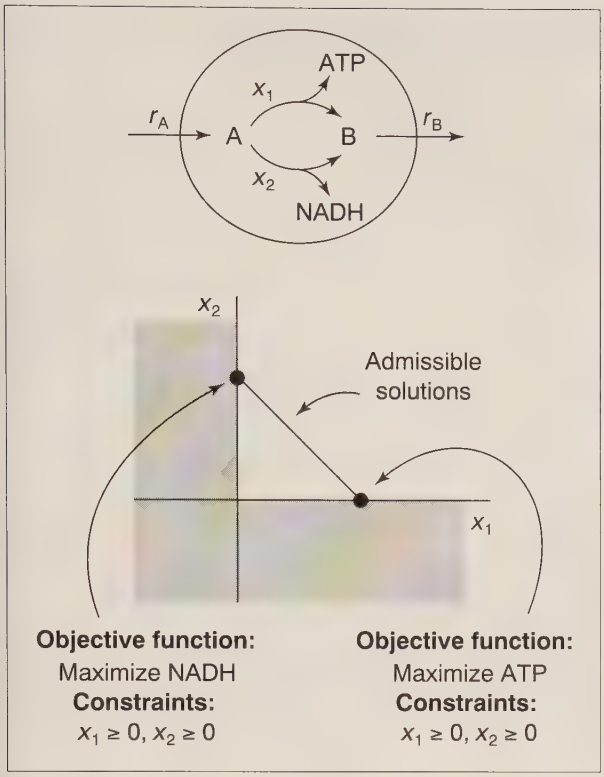


Figure 4
Linear-optimization techniques to estimate fluxes in underdetermined metabolic networks.

In this case, the constraint 'maximize NADH and NADPH production' would theoretically result in an estimated flux distribution that is similar to the experimentally determined fluxes. Similarly, chemical decouplers of oxidative phosphorylation, such as 2,4-dinitrophenol, will lower the activity of the TCA cycle. Fluxes determined from mass balances supplemented with data from isotopic-tracer methods, combined with fluxes estimated from mass balances supplemented with different theoretical constraints, may lead to a fundamental understanding of the validity of the assumptions made previously[9,15,17–20]. In addition, the combined information from mass-balancing techniques and isotopic-tracer experiments will allow the testing of the consistency of both methods, as their combination produces overdetermination of networks. This is relevant, because the use of isotope tracers for the determination of fluxes is an unsolved problem[28].

In addition, there are alternatives that do not require the demanding effort of isotopic-tracer studies and may help to solve the observability problems of rank-deficient metabolic networks. First, various studies have reported quantitative flux data for entire metabolic networks, determined using a set of isotopic tracers[4,29]. From these data (vector x), the extracellular metabolic rates (vector r) can easily be determined using the equation in Box 1. Subsequently, the constraints determined by the mass balances and the metabolic rates can be calculated and various theoretical constraints can be tested for their ability to estimate the fluxes correctly. Second, the presence of certain single-enzyme reactions in metabolic networks causes singularities in the linear set of equations that describes these networks. For example, the pyruvate carboxylase and the transhydrogenase reactions directly cause observability problems. However, in some cases, these enzymes are not active[29], a feature that can be easily measured using enzymatic assays, and if no enzymatic activity is found, then that particular flux can be deleted. Third, for several organisms, the estimated flux distribution, and in particular the split ratio at the glucose-6-phophate branchpoint, has been shown to be extremely sensitive to the NAD(P)H balance[7,18]. As this balance is mainly dependent on the rate of oxygen uptake, an improvement in the accuracy of this measurement may lead to completely observable metabolic networks. Additionally, a weighted least-squares solution could be used to account for uncertainties in mass balances such as the NAD(P)H balance.

Outlook

The near future will show whether investigations (such as some of those suggested here) will lead to the development of a universally valid method for the measurement of intracellular fluxes. In the past, it has been regarded as unwise to make generalizations about the metabolic role of futile cycles because of the lack of available experimental flux data under *in vivo* conditions[22], but this will not be the case for other metabolic cycles such as the pentose shunt, the malate shunt and the TCA cycle. Because metabolic pathways have become targets for genetic and biochemical engineers, the number of isotopic-tracer studies carried out under well-defined conditions will increase. The resulting data may provide a basis for a quantitative understanding of metabolism and help to find missing constraints. However, until then, isotopic-tracer experiments remain indispensable for the quantification of fluxes in cyclic metabolic pathways.

Other applications, such as rational medium design[10], elucidation of metabolic and toxicological effects without using radioactive or stable isotopes, rapid quantitative biochemical studies and the determination of metabolic-control coefficients[11] (for a recent review of the role of metabolic control analysis in rational bioengineering, see Ref. 30) will also benefit from such a breakthrough. In addition, it should be possible to control fermentation processes at the level of intracellular reactions by calculating the metabolic fluxes from on-line measurements of only a few key metabolites. The experimental validation of theoretical constraints will extend our knowledge of the metabolic strategy of various cell lines. Likewise, such constraints can be used as a link between genetics and physiology[13] and thus help to predict phenotypes from genome data banks.

Acknowledgements

We thank Michael Ibba for critical reading of the manuscript and Bram Timmerarends for the design of Fig. 4.

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Heterologous biopharmaceutical protein expression in *Streptomyces*

Craig Binnie, J. Douglas Cossar and Donald I. H. Stewart

The commercial production of human proteins in recombinant microorganisms for therapeutic use is well established. Systems have been developed to exploit the natural ability of certain bacteria to secrete properly folded, bioactive proteins into the extracellular medium. The streptomycetes are a relatively well-characterized group of nonpathogenic filamentous bacteria that have the capacity to secrete large amounts of protein. In particular, *Streptomyces lividans* has the ability to secrete human proteins at a commercially viable level, thanks to relatively well-established plasmid-based expression systems, a high-biomass fermentation process and a low level of endogenous protease activity.

Streptomyces are Gram-positive, spore-forming soil microorganisms that exhibit a complex morphological and physiological life-cycle on solid media. They normally grow in a mycelial manner, that is, as branched filaments lacking internal septa. Nutrient deprivation usually triggers the sporulation pathway, resulting in the formation of aerial mycelium and dormant spores. Accompanying these morphological changes is the production of a variety of secondary metabolites, many of which have potent biological activity. The streptomycetes are most often considered as a vast reservoir of natural products with diverse activities, including antimicrobial, antifungal and immunosuppressive compounds, rather than as hosts for the heterologous production of proteins of human therapeutic value. However, considerable research and development work has been undertaken during the past decade, mainly within the biotechnology industry, with the aim of exploiting the natural ability of certain species of *Streptomyces* to secrete proteins with remarkable efficiency.

When the microbial expression of recombinant proteins is discussed, *Escherichia coli* is usually assumed to be the host organism. The dominance of *E. coli* in this field is a reflection of the depth of information on its genetic and biochemical systems generated over many decades of research, both academic and industrial. However, this has not deterred the search for alternative strategies for the large-scale production of recombinant proteins with human therapeutic value. Of particular interest is the production of a protein in a soluble form but still with the appropriate tertiary structure for biological activity. Many human proteins do not require glycosylation for activity, and nonglycosylated forms of human proteins can be generated via bacterial expression and secretion. Eukaryotic microorganisms, such as *Saccharomyces cerevisiae* and *Pichia pastoris*, have been used for the secretion of glycosylated proteins into the extracellular medium. However, protein glycosylation in yeast differs significantly from that found in humans, with the result that, in many cases, the polysaccharide moieties must be removed in order to avoid immunogenicity or other adverse clinical effects. However, bacterial secretion can produce a nonglycosylated protein that is generally nonimmunogenic and still bioactive. The purpose of this review is to present the rationale for considering *Streptomyces* as a potential bacterial host for the production of heterologous proteins.

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Why choose *Streptomyces*?

The ease of large-scale cultivation and the availability of genetically well-controlled expression systems for microorganisms such as *E. coli* are factors that have appealed to the biopharmaceutical industry when strategic decisions are made to develop processes for the manufacture of certain human proteins for therapeutic application. *E. coli* is probably unsurpassed in its ability to generate product in high yield within the bacterial cytoplasm. However, the protein is usually

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PII: S0167-7799(97)01062-7

Glossary	
TNFα	tumour necrosis factor alpha
GM-CSF	granulocyte macrophage-colony-stimulating factor
IL-1β	interleukin 1-beta
IL-3	interleukin 3
IL-6	interleukin 6
EPO	erythropoietin
SCF	stem cell factor
GRAS	generally regarded as safe

produced in an intracellular, insoluble, denatured state, in the form of inclusion bodies. The process of recovering a properly folded and bioactive protein then involves the disruption of cells, isolation of inclusion bodies, renaturation of the desired protein into an active conformation and purification of the target protein from contaminating host proteins. This exercise can cause many practical difficulties, for the efficiency of recovery of a fully functional protein is also related to the relative efficiency of refolding, which varies from protein to protein. In addition, intracellularly expressed proteins usually contain an amino-terminal methionine residue, which has to be removed. Safety concerns arising from the use of a bacterial host known to produce lipopolysaccharide pyrogens must also be addressed during the manufacturing process.

The strategy of secreting a heterologous protein into the periplasm or directly into the extracellular medium represents an attractive alternative method for overcoming some of the problems encountered by intracellular production. The periplasmic compartment in *E. coli* offers the appropriate redox environment for the generation of disulphide bonds, and the process of secretion itself is more likely to direct correct protein folding. This has been demonstrated in *E. coli* using the *omp*A signal sequence[1]. However, this approach appears to have limited application for large-scale production, due mainly to inefficient secretion that results in low yields of the final product. Indeed, 'efficient' secretion in *E. coli* is often associated with the production of periplasmic inclusion bodies. Well-characterized nonpathogenic (generally regarded as safe, or 'GRAS') Gram-positive microorganisms lacking an outer membrane have also been utilized as secretion hosts. The bacilli and actinomycetes are well known for their capacity to secrete large amounts of high molecular weight proteins (e.g. amylases, proteases) into the extracellular medium. *Bacillus subtilis* has been assessed by many groups as a potential host for heterologous protein expression, but high levels of multiple endogenous proteases have, in general, limited the yield of intact heterologous protein secreted into the medium[2]. The construction of protease-deficient *B. subtilis* strains[3] or the use of non-proteolytic species such as *Bacillus brevis*[4,5] have improved the prospects of achieving a high (commercially viable) level of expression of heterologous proteins in *Bacillus*, but the reported yields have so far been relatively low[2].

The ability of *Streptomyces* to secrete heterologous proteins was first reviewed by Chang and Chang[6], who utilized an expression system in *Streptomyces lividans* harbouring the stable, well-characterized multicopy plasmid pIJ702 (Ref. 7) with additional transcriptional, translational and secretory elements drawn from separate species of *Streptomyces*. The strong constitutive promoters from either the *Streptomyces fradiae* aminoglycoside phosphotransferase (*aph*) gene or the erythromycin resistance gene (*erm*E) from *Saccharopolyspora erythraea* were utilized in combination with fragments encoding signal peptides from the *Streptomyces antibioticus* tyrosinase gene (*mel*C) or the *Streptomyces limosus* α-amylase gene (*aml*). Biologically active human tumour-necrosis-factor alpha (TNFα) was produced in this system at levels of up to 20 mg l^{-1}, using a mutant version of the *erm*E promoter[8] (known as *erm*E* or *erm*Eup) in combination with the *mel*C signal peptide. Since Chang and Chang's study in 1988, several reviews have been published on the secretion of heterologous proteins in *Streptomyces* that have further illustrated the general utility and versatility of this genus as a host for expression of bioactive proteins[9–14].

Choice of host species

Although *Streptomyces coelicolor* A3(2) is the most extensively characterized species within the genus, its close relative *S. lividans* 66 is almost exclusively the species of choice as a host for heterologous protein production. Unfortunately, *S. coelicolor* A3(2) is typical of many streptomycetes in that it contains a very powerful restriction system that acts as a barrier to the entry and propagation of *E. coli*-derived shuttle vectors. However, *S. lividans* lacks this system, and thus has been extensively exploited[15]. In addition, *S. lividans* is also unlike most other streptomycetes in that it generally displays a very low level of endogenous extracellular proteolytic activity. However, it still retains the ability to produce large quantities of extracellular protein[16].

Levels of production of biopharmaceutical proteins

Examples of the yields of some heterologous proteins expressed in *S. lividans* are presented in Table 1. The yields reported were obtained using a shake-flask process [except for the sCD4 receptor (see Table 1 and Ref. 16)], and consequently represent the lower end of the potential range. An illustration of the relative levels and purity of a variety of proteins secreted by recombinant *S. lividans* is shown in Fig. 1. The example of sCD4 receptor expression in *S. lividans* is particularly important in the context of this review, as it represents a case in which the expression rate and product characteristics [such as protein solubility, proper formation of disulphide bonds (as measured by reactivity with conformationally active monoclonal antibodies) and bioactivity] are clearly superior to that documented for the same protein produced in other hosts, such as *E. coli* and Chinese hamster ovary cells.

Plasmid systems

Most of the plasmids developed for high-level expression in *S. lividans* are *Streptomyces–E. coli* shuttle vectors, based on derivatives of the multicopy pIJ101 replicon[7], and exhibit a broad host range in *Streptomyces*. The major antibiotic-resistance marker used for recombinant selection in *S. lividans* is the thiostrepton-resistance gene from *Streptomyces azureus*[17]. This plasmid is extremely stable, even in the absence of selection[18]. It is generally introduced into the host by protoplast transformation, and efficient regeneration methods are available[19].

Transcription signals

DNA fragments with promoter activity in *Streptomyces* can be classified into two main categories[20]. The first group appears to conform at least in part to a canonical *E. coli* σ^{70}-type, containing consensus sequences located at defined distances (centred around 10 and/or 35 bases) upstream of the transcription start-point. Examples of this group of promoters include several that are functional in both *S. lividans* and *E. coli* [e.g. *XP55-p* (Ref. 21) and *SEP2* (Ref. 22)]. The second, larger, group of sequences identified as promoters in *Streptomyces* generally do not function in *E. coli*. Some have been shown to contain multiple recognition motifs, either overlapping or in tandem, for both the major and minor forms of RNA polymerase holoenzymes (differing in sigma-factor content), which are responsible for directing specific developmentally, temporally or nutritionally regulated patterns of gene expression. The *aph* promoter (mentioned above) is one of the most powerful constitutive promoters available and it has been used extensively to direct transcription of foreign genes in *S. lividans*[6,13]. Transcriptional analysis of this promoter[23] revealed two tandem nonoverlapping sequence motifs, but little is known about their relative strength *in vivo* nor which of the RNA polymerase holoenzyme forms are used for their expression. The *erm*Eup promoter is a similar constitutive promoter that has been used to direct high-level expression of foreign genes[6,24]. The promoter from the *Streptomyces longisporus* STI-II gene (encoding a secreted protease inhibitor) has also been used to direct the expression in *S. lividans* of soluble human CD4-receptor derivatives at up to 300 mg l^{-1} (Table 1)[25]. Promoters are available that can be regulated, such as those induced by galactose[26] or thiostrepton[27], but these have not yet been developed for use in an expression system. The *veg* promoter of *B. subtilis* is transcribed efficiently in *S. lividans*[28] but it has also not been developed for the expression of heterologous proteins.

The efficient termination of transcription has also been shown to be a factor contributing to optimal protein expression[29]. In general, transcription terminators of *Streptomyces* genes are typical of those found in other bacteria, containing long, imperfect, inverted repeat nucleotide sequences with the potential to form hairpin structures, akin to the rho-independent terminators of *E. coli*. However, they generally lack the extended thymidine-rich sequence typical of rho-independent terminators.

Table 1. Examples of human proteins expressed and secreted by *Streptomyces lividans*

Gene	Yield
sCD4 receptor derivative[25]	>300 mg l^{-1}
Proinsulin[39]	20–100 mg l^{-1}
Tumour necrosis factor α (Ref. 6)	20 mg l^{-1}
Granulocyte macrophage-colony stimulating factor (Ref. 46)	10–15 mg l^{-1}
Interleukin 1β (Ref. 47)	1–5 mg l^{-1}
Interleukin 3 (Ref. 13)	80–100 mg l^{-1}

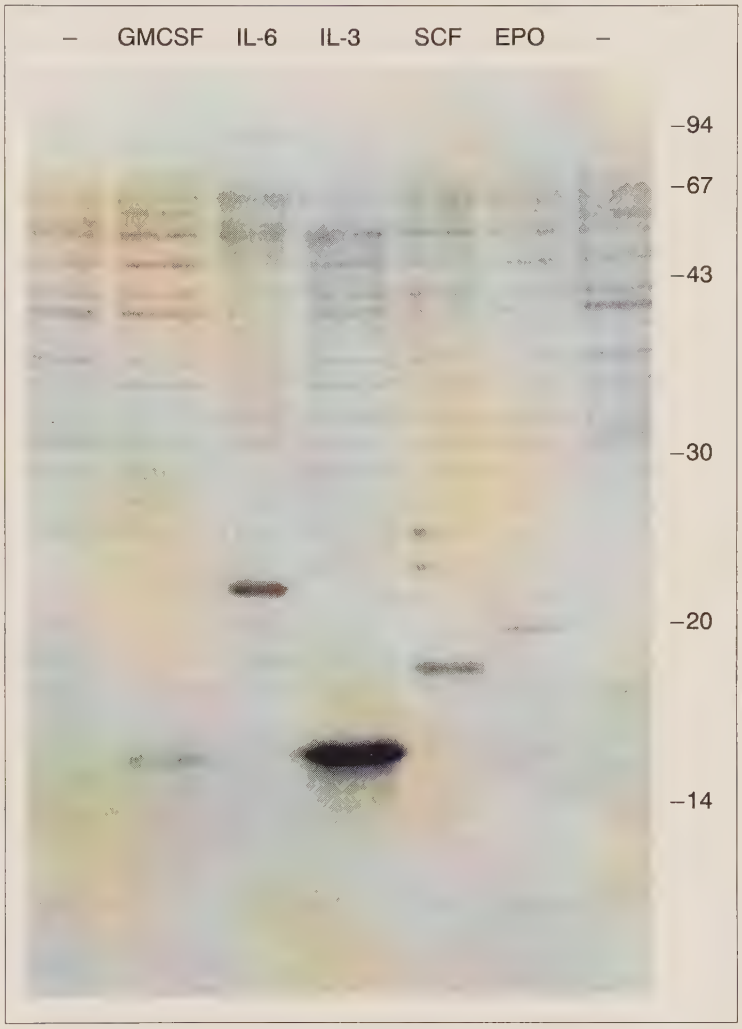

Figure 1
SDS-PAGE analysis of examples of human proteins secreted by recombinant *S. lividans*. The unmarked lanes represent a vector control culture, and the relative positions of molecular weight standards in kDa are as shown. All lanes contain samples of unpurified cell-free broth from transformants grown in shake flask culture for 18 h (reproduced from Ref. 13 with permission).

Translation signals

A comprehensive analysis[20] of known *Streptomyces* mRNA leader sequences illustrated clear differences

to the *E. coli* and *B. subtilis* paradigms. Many sequences proximal to open reading frames have been found that display significant complementarity to the 3′ end of the 16S ribosomal RNA in *S. lividans*, clearly representing the typical ribosome binding site (Shine–Dalgarno motif). In stark contrast to other bacteria, a significant fraction of *Streptomyces* genes are apparently translated from leaderless transcripts, in which the start point of transcription coincides with the first base of the translation initiation codon[23]. Perhaps surprisingly, this class of genes includes *aph* and *erm*E, whose leaderless promoter sequences have been utilized to a greater extent than leadered versions in *Streptomyces* protein expression systems[6,13]. However, leader mRNA in *Streptomyces*, where it exists, is much longer and more variable in length than in *E. coli*, often containing sites of significant predicted secondary structure, which are characteristic targets for regulatory proteins involved in modulating gene expression in bacteria.

Codon usage

One of the hallmarks of the *Streptomyces* genome is the high (>70%) GC content, a phenomenon that is either the cause or the consequence of a highly biased codon-usage pattern, in which GC-rich codons are overrepresented. For example, the TTA and CTA leucine codons are confined to a small number of known *Streptomyces* genes. The impact of favoured and unfavoured codon usage on the expression of heterologous genes in different bacteria is still debatable. However, there is some evidence of improved expression in *E. coli* when certain rare codons are changed to preferred codons, or of a decrease in expression when the codon change occurs in the reverse direction[29]. This phenomenon is thought to be related to the relative levels of the intracellular pool of charged transfer RNA molecules, which are low for rarely used codons and high for frequently used codons. Ueda *et al.* have shown that, in *S. lividans*, when certain preferred codons for leucine in a *Streptomyces* gene are changed to TTA, gene expression is decreased[30]. In contrast, when five consecutive minor codons occurring close to the 5′ end of the mouse TNFα gene were changed to preferred codons, a 10^4-fold drop in protein secretion was observed[31]. The basis for this phenomenon is likely to be multifactorial, probably involving sequence context and positional frequency, among other factors. The influence of codon changes on mRNA secondary structure may have a profound effect on gene expression and merits further study. In summary, no unambiguous rules governing the probability of rare codons affecting overall protein expression have been formulated, especially in *Streptomyces*. For now, the more empirical approach of targeted random mutagenesis (i.e. mutating defined regions randomly) is more likely to result in an improved expression system.

Secretion signals and host factors

Signal peptides in *Streptomyces*, used to direct export of protein from the cell, conform to the general rules elucidated for bacterial secretion systems[32]. However, the signal peptide in *Streptomyces* is generally longer than in *E. coli* or *B. subtilis*. A short stretch of positively charged hydrophilic amino acids (e.g. Arg, Lys) at the amino terminus is usually followed by a long alpha-helical 'core' of hydrophobic amino acids (e.g. Leu, Ala, Val), a helix-breaking residue (e.g. Pro) and a short carboxy-terminal sequence motif containing small amino acids (e.g. Ala, Asn) that is recognized and has its peptide bonds cleaved by a bacterial-type-I signal peptidase. The influence of altering the net charge of the signal peptide on secretion efficiency in *S. lividans* has been shown to be significant and worthy of further study[33]. Cleavage of the peptide bond between amino acid residues at the −1 and +1 position of the mature secreted protein occurs immediately after membrane translocation, a process that is presumably facilitated by both intracellular chaperones and membrane proteins, homologous to the many secretion-facilitator proteins identified in the general secretory pathway of *E. coli* and *B. subtilis*. *Streptomyces* genes homologous to *sec*A, E, Y, D, F[34], *gro*ES/*gro*EL[35] and *dna*K[36] have been identified, but their contribution to the secretion of heterologous proteins is presently unknown.

The efficiency of disulphide bond formation is one other factor that can influence the rate of correct folding of proteins during and after secretion. Accurate folding of secreted proteins in the periplasm of *E. coli* is not merely due to intramolecular associations but is catalysed by host proteins that can promote disulphide-bond formation. No homologues of such proteins are yet known in *Streptomyces*, but their presence in other Gram-positive bacteria with a highly developed secretion apparatus (such as *B. brevis*[37]) would indicate that they also exist in *Streptomyces*.

Other strategies for facilitating secretion of a poorly expressed eukaryotic protein in bacteria include fusion of its gene downstream of a well-expressed native gene. There are few documented examples of this approach being attempted in *Streptomyces*, but at least two reports[38,39] have illustrated its potential utility.

The most well developed *Streptomyces* expression systems generally utilize signal peptides from well-expressed native genes, including those encoding *S. griseus* protease B (Ref. 40), subtilisin-inhibitor proteins from *S. albogriseolus*[38], *S. venezuelae*[41] and *S. longisporus*[25], and tendamistat, an alpha-amylase inhibitor from *S. tendae*[42]. The enzyme-inhibitor-derived signals can work well in conjunction with their cognate promoter sequences, possibly suggesting the existence of an evolutionary co-optimization process, in which transcriptional and translational signals and/or sequences have been coselected for high-level secretion.

Extracellular processing events

The result of Chang and Chang[6] suggested that the TNFα secreted by *S. lividans* was subject to processing by host aminopeptidase activity during the

secretion process, resulting in the formation of a protein lacking the first four amino acids of mature TNFα. Amino-terminal processing of secreted proteins by cosecreted host aminopeptidases is probably sequence-dependent, and has been noted for some heterologous proteins[43] and for some native *Streptomyces* proteins[25]. The judicious positioning of specific peptide leaders at the amino terminus of heterologous proteins provides substrates for well-characterized *Streptomyces* extracellular aminopeptidases, a strategy that has been applied successfully to improve the efficiency of protein secretion and recovery in *S. lividans*[16].

Fermentation studies

Very little data has been reported for the large-scale production of recombinant proteins in *Streptomyces*. However, it is possible to obtain a high level of biomass and protein yield at bench-scale within an acceptable time frame[25]. The established approach (using a balanced medium in a fed-batch fermentation to improve the total biomass, and avoiding physiological stress by maintenance of culture pH and oxygen content), coupled with standard methods of reducing extracellular protease production, is an obvious route for optimizing the production of intact heterologous protein[44]. However, the rheological characteristics of a high-biomass culture of a filamentous microorganism pose an additional challenge in the design of large-scale production processes[45].

Outlook

S. lividans has been shown to be a commercially viable host for the production of a variety of active biopharmaceutical proteins. Nevertheless, the use of co-optimized combinations of strong promoters, signals and leader sequences in combination with the heterologous genes is the most likely route to greater expression levels in this host. Further elucidation of the subtle interactions occurring at the nucleotide level between these elements and the structural gene may lead to the development of strategies for more efficient expression of heterologous genes. However, the overall recovery of certain biopharmaceutically active proteins is also affected by protein-specific factors, including the rate and efficiency of membrane translocation and the relative resistance to host proteolytic processing during and after secretion. The further development of mutant strains deficient in extracellular proteases should improve the quality and/or quantity of heterologous protein expression. Alternatively, an understanding of the substrate specificity of secreted aminopeptidases could be exploited to design specific inhibitors, to diminish postsecretory degradation during fermentation. The characterization and genetic manipulation (by deletion or overexpression) of genes encoding other secretion-facilitating proteins should also provide the molecular tools required to develop *Streptomyces* as a general bacterial host for the expression of a variety of biopharmaceutical proteins.

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Transgenic animals as models of neurodegenerative diseases in humans

Franz Theuring, Markus Thunecke, Ulrich Kosciessa and Jonathan D. Turner

Neurodegenerative diseases are of major socioeconomic importance and represent an enormous challenge for the scientific and medical communities. Advances in molecular genetics during the past decade have begun to provide approaches for the establishment of animal models for these disorders using transgenic technology. Their analysis will lead to better understanding of disease pathogenesis and will be invaluable for the identification of novel diagnostic and therapeutic agents. With the current pace of genomic research, the generation of transgenic animal models, reproducing in full the pathology and symptoms of even complex disorders such as Alzheimer's disease, must now be considered achievable.

Advances in the understanding of the genetics of hereditary neurodegenerative disorders and of the risk factors associated with the occurrence of sporadic disease have provided the basis for the development of the animal models required for the elucidation of their pathogenesis and for the development of rational therapies. In the past decade, intensive application of transgenic technology has led to the establishment of a number of rodent models that mirror different aspects of several of the most important neurodegenerative diseases.

Transgenic animal models of Alzheimer's disease

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases in the developed world. AD is characterized by two major pathological hallmarks: amyloid plaques, which contain mainly aggregated Aβ peptide (βA4), a 39–43 amino acid peptide derived from a precursor protein (amyloid precursor protein, APP) by proteolytic cleavage; and neurofibrillary tangles (NFTs), characterized by the presence of hyperphosphorylated tau, a microtubule associated protein. None of these pathological signs occurs spontaneously in rodents.

To date, three genes have been found to be associated with familial forms of AD (FAD). These include the APP gene on chromosome 21 and the recently identified presenilin (PS) genes, PS-1 on chromosome 14 and PS-2 on chromosome 1 (Ref. 1). Mutations in these genes have all been shown to elicit changes in the proteolysis of APP, leading either to excessive generation of Aβ, or to overproduction of the more amyloidogenic forms of the peptide (Fig. 1). These mutations also lead to an earlier and more profound appearance of pathology and symptoms of the disease,

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PII: S0167-7799(97)01066-4

which is otherwise indistinguishable from sporadic AD. In the sporadic disorder, the presence of the ϵ4 alleles of the Apolipoprotein E (ApoE) gene has been demonstrated to be a risk factor for the disease. These findings have provided entry points to attempt to model the disease phenotype in transgenic rodents.

Various wild-type and mutated cDNA and yeast artificial chromosome (YAC) transgene constructs have been used in attempts to elicit overexpression of human APP isoforms. Using homologous recombination in embryonic stem (ES) cells and the subsequent generation of chimeric mice, attempts have been made to delete the endogenous APP genes (knockout) or to replace parts of the endogenous gene sequence with that of the human gene (knockout–knockin) in attempts to gain insight into APP-gene function in knockout animals or to humanize endogenous mouse APP (see Table 1 for a list of the most prominent studies).

Despite intensive efforts, no approach was successful in generating Alzheimer-like pathology until the report of the 'Athena mouse'[2], which used an APP minigene construct harbouring the human APP introns 6, 7 and 8 and the 717 FAD mutation (which leads to increased production of the more amyloidogenic Aβ 1–42) under the control of the strong, neurone-specific PDGF-β promoter. Several lines showing amyloid and gliotic pathology were generated. Another model, using the prion promoter and a human APP cDNA construct carrying the 670/71 FAD (Swedish) mutation that causes an eight- to tenfold increase in Aβ production, was also shown to generate amyloid plaques and the associated glial pathology[3]. These two models imply that very high levels of overexpression of transgenic APP are necessary to elicit amyloid deposition. This may also explain why the introduction of the whole human gene locus, including all its regulatory sequences, using the YAC approach, and the humanization of the rodent APP gene by knockout–knockin have not been successful in eliciting amyloid pathology. It is not yet clear whether the introduction of a FAD mutation into the transgene is needed or not.

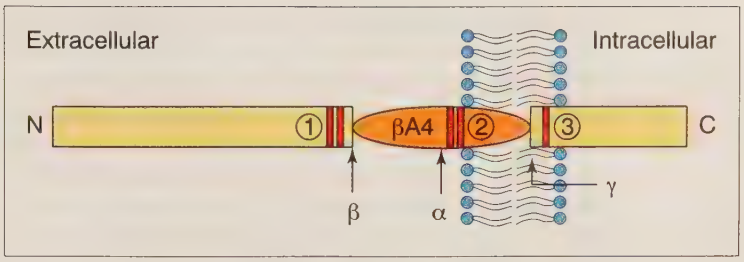

Figure 1

The major component of senile plaques in AD, the βA4 peptide is generated from the transmembranous amyloid precursor protein by the action of two protease activities (β- and γ-secretases), although the bulk of normal constitutive processing of APP occurs by α-secretase cleavage, which yields nonamyloidogenic products. In familial Alzheimer's disease, mutations in the APP gene have been found to cluster around the cleavage sites (vertical bands: 1, Swedish mutation; 2, Dutch or AD mutation; 3, London mutation) and have been shown to interfere with APP processing, so causing increases in the amount of βA4 and/or in the more amyloidogenic, longer forms of βA4 generated[1]. Because these mutations lead to earlier onset of the disease and, in some cases, to more pronounced pathology, their introduction into transgenic animals has been the primary target in attempts to generate rodent models of the disease.

A third model uses PS-1 cDNA constructs under the control of strong neurone-specific promoters[4,15]. Here, animals expressing PS-1 mutations produced larger amounts of the most amyloidogenic Aβ form (Aβ 1-42), in accordance with observations from patients. In contrast, transgenic expression of unmutated PS-1 showed almost no effect on Aβ levels. Until now, however, no plaque formation has been reported, which may be due to the reduced amyloidogenicity of the mouse Aβ compared with the human forms

Table 1. Alzheimer's disease and transgenic mouse models

Construct (promoter/gene)	Pathology	Ref.
PDGF-β/APP-minigene V717F	Amyloid plaques, gliotic changes	2
PrP/APP KM670/71NL	Amyloid plaques, gliotic and behavioural changes	3
PDGF-β/PS-1	Aβ 1–42 increase	4
Thy-1/tau	None	5
ApoE ko	None	6
NSE/APP	None	7
NF-L/Aβ	Neurodegeneration, apoptotic cell death	8
Thy-l/APP mutant at α-site	Brain morphology changes, seizures, early death	9
APP-YAC	None	10
APP-YAC	None	11
APP ko	Behavioural and anatomical changes	12
APP ko	None	13
APP ko–ki	None	14
PrP-PS1 X PrP/APP KM670/71NL	Aβ 1–42 increase	15

Abbreviations: NSE, neurone-specific enolase; APP, amyloid precursor protein; NF-L, neurofilament, light chain; PDGF, platelet-derived growth factor; PrP, prion protein; YAC, yeast artificial chromosome; ko, knock out; ki, knock in; ApoE, apolipoprotein E; PS, presenilin.

expressed by the APP-cDNA-expressing transgenics. An important next step will be to crossbreed these animals with mice showing amyloid pathology, to determine whether the presence of mutated presenilins affects the onset, development and severity of the pathology in the manner predicted from the FAD cases. All of these models will be of use, and one at least has already been used, for the characterization *in vivo* of therapeutics interfering with Aβ generation, and for the assessment of *in vivo* diagnostics. The first two models may also be of use for testing compounds for their ability to prevent Aβ aggregation.

Although some show convincing amyloid pathology, largely occurring in those brain regions primarily afflicted in AD, none of these APP or PS-1 models showed signs of NFT development, and so far only very unconvincing influences on behaviour have been reported. As such, they can only be considered partial models of AD. While expression of human Tau cDNA under the control of the human Thy-1 promoter led to the appearance of hyperphosphorylated tau epitopes similar to those in AD brain in nerve cell bodies, axons and dendrites, no NFT formation was detectable[5]. Genis and co-workers reported AD-like tau phosphorylation in ApoE-deficient mice, which may link ApoE and the neuronal cytoskeleton and indicates a possible function of ApoE in neuroprotection[6]. However, the presence of hyperphosphorylated tau epitopes as precursors of neurofibrillary pathology should be interpreted with caution until they have been associated definitively with the aetiology of the disease.

The development of a transgenic model of AD has been the most intensively pursued goal in transgenics research in recent years, and has yielded the largest number of disappointments. Reasons for the many failures are still not clear, but a frequent cause has undoubtedly been the lack of sufficiently strong transgenic-protein expression, possibly coupled with inappropriate cellular expression. The failure to generate the full AD pathology in transgenic animals before now may lie in the difficulty in recapitulating, in the lifespan of the laboratory rodent, a chronic disease process that progresses over decades in man. Other possibilities are that the strategies employed may still be too simple to mimic the, probably heterogeneous, aetiology of the sporadic disease, or that the regulation of processing of the human proteins differs in rodents and man.

Despite these difficulties, the recent generation of animals showing AD-like amyloid pathology provides great hope for the future (Table 1). Drug-discovery programmes in industry throughout the world are generating compounds capable of inhibiting either Aβ generation or aggregation, or the proinflammatory or directly neurotoxic consequences of Aβ deposition in the brain. Such drugs will have utility in prevention of AD or in inhibition of its progression. The partial models currently available have already found application in the preclinical evaluation of certain of these approaches. Crossbreeding of the strains already produced may give more insight into the disease process and elicit additional aspects of pathology, while transgenic manipulation of additional factors possibly involved in the aetiology of AD (e.g. candidate proteases for the amyloidogenic processing of APP) and crossbreeding these into the existing strains will confirm therapeutic targets and enhance the validity of rodent models of this devastating disorder.

Transgenic animal models of the prion diseases

The transmissible spongiform encephalopathies (TSEs) comprise a group of slow degenerative diseases of the central nervous system (CNS) such as Kuru, Gerstmann–Sträussler–Scheinker disease (GSS), Creutzfeldt–Jakob disease (CJD) and fatal familial insomnia (FFI) in man, scrapie in sheep and bovine spongiform encephalopathy (BSE, 'mad cow disease') in cattle. These diseases manifest as infectious, genetic and sporadic disorders[16] and can be transmitted among mammals by the pathogenic agent designated a 'prion'[17], which is proposed to lack nucleic acid and consist only of proteins (for reviews see Refs 18–20). The 'protein only' hypothesis proposes that the prion is identical with the scrapie form of the prion protein (PrPSc), a modified form of the cellular form of the prion protein (PrPc), which is a normal host protein found predominantly on the surface of neurones and is encoded by a single exon of a normal cellular gene termed *Prn-p* in the mouse and *PRNP* in humans. PrPSc is a protease-resistant form of PrPc that readily forms aggregates, preceding neuronal loss and spongiform changes. PrPc is proposed to be the substrate for PrPSc, which, when introduced into a normal cell, causes its conversion into new PrPSc and initiates a vicious circle[19,20].

Most, if not all, familial forms of human TSEs are linked to pathogenic mutations in the human *PRNP* gene[21], which allow spontaneous conversion of PrPc into PrPSc and development of the disease within the average lifetime of the individual. Mice expressing a murine PrP transgene with the leucine substitution [Tg(MoPrP–P101L)], corresponding to the human GSS mutation at codon 102, spontaneously develop a neurological disease with spongiform degeneration[22], but PrPSc was not detected. Additional lines of Tg(MoPrP–P101L) mice faithfully recapitulated virtually all features of human GSS (P102L)[23], clearly demonstrating that the P101L mutation, and not mere overexpression of PrP, causes the clinical and neuropathological features of GSS. Although successful transmission of CNS pathology from spontaneously affected transgenic mice to unaffected transgenic mice after intracerebral inoculation was observed, again no PrPSc was detectable.

Transgenic mice expressing hamster PrP have been generated to test the hypothesis that differences in PrP gene sequence are responsible for the reduced efficiency of transmission between species. Inoculation of these mice with hamster prions resulted in loss of the species barrier, followed by an abbreviated incubation time inversely proportional to the level of hamster PrPc in the brains of transgenic mice[24–26].

The 'protein only' hypothesis predicts that in the absence of PrPc, mice should be resistant to prion infection. To test this, knockout mice have been generated in which the *Prn-p* gene was deleted by homologous recombination in ES cells[27]. No pathology or disease is found following prion infection in these animals, which lack the ability to make PrPc and therefore cannot produce PrPSc[28]. The reduced, but persistent, vulnerability in heterozygous animals demonstrates that susceptibility to prion disease is a function of PrPc levels in the host[29]. The return of vulnerability following transgenic reconstitution of PrP in these animals further confirms this notion[30].

In an elegant set of experiments, Brandner and colleagues grafted neural tissue from transgenic mice overexpressing PrPc into the brain of PrP-knockout mice[31]. Following intracerebral inoculation with infectious prions, high levels of PrPSc deposits and infectivity were found in the grafted tissue, which went on to develop severe spongiform degeneration. However, adjacent host-derived PrP-deficient neural tissue showed no histopathological changes even though PrPSc migrated out of the graft and into the host brain. These results confirm that PrP-deficient cells are not susceptible to PrPSc-induced pathology and imply that the availability of PrPc, and not the deposition of PrPSc, is directly linked to prion histopathology[32].

Most recent transgenic studies have been directed to the investigation of the molecular basis of prion strain specificity (that is, why different prion strains elicit different disease phenotypes). Thus the mouse PrP knockout, reconstituted with human PRNP, is being used to examine the transmission of different CJD prions, and should deliver much-needed information regarding the association of 'new variant' CJD with BSE[33].

In conclusion, the application of transgenic technology to the prion disorders is the most successful example to date of its use for the elucidation of disease pathophysiology in a neurodegenerative disorder.

Transgenic animal models of amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS), also called Lou Gehrig's disease after the famous baseball player who suffered from it, is characterized by a specific loss of motor neurones in the brain and spinal cord. Clinically, the degeneration of motor neurones is accompanied by a secondary denervation atrophy of skeletal muscles leading ultimately to complete paralysis (for a recent review, see Ref. 34). The disease occurs in both sporadic and familial forms (FALS), with FALS accounting for approximately 10% of all cases[35]. The exact cause of the sporadic forms remains unknown, although several theories exist. For example, the excitotoxic effect of a pathological, excessive release of glutamate at synapses on their cell bodies may play a role in the degeneration of motor neurones[36]. Alternatively, the enhanced levels of circulating IgG immune complexes in ALS suggest an immunological dysfunction[37]. The most important clues, however, come from the genetic evidence that mutations in the Cu/Zn superoxide dismutase 1 (*SOD*1) gene at chromosome 21 are the cause of the disease in about 20% of FALS cases[38].

The generation of transgenic mice as models for human ALS has proved to be another very successful application of transgenic technology. Three groups generated transgenic mice by introducing the complete human or murine *SOD*1 gene (wild-type and mutated forms) into the mouse genome[38–41]. The animals developed several clinical signs of ALS, such as progressive weakness, muscle atrophy and, in the most severe cases, paralysis. The specificity of these results was underlined by the fact that mice expressing wild-type *SOD*1 showed no signs of disease. A histopathological examination revealed vacuolar changes in the dendrites, cell bodies and axons of motor neurones[38–42]. Neurofilamentous aggregations, another characteristic feature of human ALS, were also observed in mice expressing the Gly-93→Ala mutation, thus linking *SOD*1 mutations with the cytoskeletal pathology observed in human ALS[38].

The molecular basis for the toxicity of the mutant SOD-1 protein is not well understood, although the results of the transgenic experiments clearly indicate that it is not caused by a change in SOD-1 activity *per se*. One possible explanation of mutant SOD1 toxicity could be aberrant interaction with other proteins, such as lysyl-tRNA synthetase and the translocon-associated-protein delta, both of which have been shown to interact with mutated, but not wild type, SOD-1[43]. Alternatively, it has been suggested that, in SOD-1 mutants, the copper cofactor of the enzyme could catalyse free radical insult, which would then damage particularly those proteins with slow turnover, such as neurofilaments (for reviews see Refs 44 and 45). Neurofilament damage would lead to impaired axonal stability and transport, and so to cell death, which could explain the selective vulnerability of the long-projection neurones of the motor system in this disorder.

The participation of neurofilaments in the aetiology of ALS was elegantly demonstrated by three studies showing that transgenic mice overexpressing the neurofilament proteins wild-type murine NF-L[46], wildtype human NF-H[47] or a mutated murine NF-L[48] all show several of the clinical and pathological features of human ALS. The animals developed abnormal accumulations of neurofilaments in specific groups of motor neurones, closely resembling the pathology of the human disease. The speculation that impaired axonal transport in vulnerable neurones might lead to axonal shrinkage and, ultimately, to neuronal death (the 'dying back' model) was supported by the finding that mice expressing the NF-H subunit display reduced axonal transport[49].

Taken together, the generation of transgenic mouse models for human ALS demonstrates the strengths of this technology in generating models that lead to a

deeper understanding of the aetiology of disease. Additionally, the presence of small-animal models enables the pharmaceutical industry to screen for compounds that might halt the progression of this progressive and fatal disease.

Transgenic animal models of trinucleotide-repeat disorders

Most of the trinucleotide-repeat disorders result from the expansion of a translated CAG repeat, leading to an elongated polyglutamine tract in the translated protein (for recent reviews see Refs 50 and 51). CAG expansions have recently been associated with a growing number of inherited movement disorders, especially inherited ataxias. The locations of the CAG expansions of five inherited movement disorders have been mapped (Table 2), the most prominent being Huntington's disease (HD), which has been mapped to the *Huntingtin* gene on chromosome 4. The normal and expanded sizes of CAG repeats in HD have been defined as CAG_{6-37} and CAG_{35-121} (the numbers indicating the length of the polyglutamine tract). The length of the CAG repeats correlates negatively with the age of onset of clinical symptoms, which include motor-coordination problems, excessive movement (chorea) and emotional disturbance. The longest repeats have been found in juvenile HD[52].

The molecular mechanisms underlying the selective neuronal death in the CAG-repeat diseases are still largely unknown, although some hypotheses exist. They are all based on the assumption that the effect of the CAG expansion is mediated at the protein level, because mice expressing a construct with a CAG-expansion only at the RNA level show no signs of disease[53]. One possibility is that CAG expansion leads to inappropriate apoptosis, because the huntingtin protein is known to be specifically cleaved during apoptosis by a cysteine protease, apopain, and this cleavage is enhanced by the presence of a polyglutamine tract[54]. Another possibility is that the presence of the polyglutamine tract modifies the interaction of huntingtin with other cellular proteins in a deleterious way and compromises the viability of certain neurones, which then die or become more vulnerable to other insults such as free radicals or excitotoxicity. Proteins for which an interaction has been demonstrated include glyceraldehyde-3-phosphate dehydrogenese, which also interacts with atrophin and ataxin, the products of the *DRPLA* and *SCA-1* genes respectively (see below), thus providing a possible link to other CAG-expansion disorders[55]. Other proteins include huntingtin-associated protein 1 and ubiquitin-conjugating enzyme (for review see Ref. 56). Much has yet to be learned of the physiological and possible pathological functions of these protein interactions.

The effect of expressing exon 1 of the human *Huntingtin* gene carrying $CAG_{115-150}$ in transgenic mice has recently been described[57]. The transgenic mice developed a progressive neurological phenotype that consisted of several movement disorders and a general loss of body weight and muscle bulk. Surprisingly, no neuropathological changes, apart from an overall reduction of brain size, were observed in these mice. Further studies with mice expressing the complete huntingtin protein with and without CAG-expansions will be needed to allow the specificity of the described phenotypes to be assessed. In this context, it is interesting to note that the authors' initial aim was the injection of a YAC containing the complete *Huntingtin* gene. Although the microinjection of YACs containing entire human genes is not trivial, the potential advantages (for example, all the regulatory elements of the human gene are present, ensuring an expression pattern in transgenic mice closely resembling the human situation) should be worth the effort.

Another strategy, the targeted disruption of the *Huntingtin* gene (knockout approach) has led to embryonic lethality in homozygous mice and to significant neuronal loss in the subthalamic nucleus in heterozygotes[58]. These results strongly suggest an essential role for this gene during development.

The other CAG-repeat diseases for which transgenic mice exist are spinocerebellar ataxia type 1 (SCA1)[59] and Machado–Joseph disease (SCA3)[60]. Transgenic mice expressing a SCA1 cDNA construct with CAG_{82} under the control of a Purkinje-cell promoter developed a progressive ataxic phenotype. Histopathological examination showed a significant loss of Purkinje cells in the brains of these mice[59].

Mice transgenic for the CAG_{79} version of the *SCA3* gene under control of the Purkinje-cell promoter developed no phenotype, whereas mice expressing

Table 2. CAG-repeat disorders and transgenic mouse models

Disease	Number of CAG repeats		Inheritance	Transgenic model
	Normal	Expanded		
Spinal and bulbar muscular atrophy	7–33	38–68	xr	no
Spinocerebellar ataxia (SCA1)	6–39	41–81	ad	yes
Huntington's disease (HD)	10–35	37–121	ad	yes
Machado–Joseph disease (SCA3)	14–44	61–81	ad	yes
Dentatorubropallidoluysian atrophy (DRLPA)	5–30	49–85	ad	no

Abbreviations: ad, autosomal dominant; xr, X-linked recessive.

only the CAG_{79} fragment of the gene driven by the same promoter developed an ataxic phenotype accompanied by a loss of Purkinje cells in the cerebellum. The phenotypes of transgenic mice expressing only the CAG-repeat part of *SCA3* or *Huntingtin*[57,60] clearly point toward toxic 'gains of function' of proteins carrying glutamine expansions.

The presence of these transgenic models will hopefully shed some light on the molecular mechanism(s) by which polyglutamine tracts lead to selective neuronal death.

Conclusions

The ability to introduce genes into the germline of mice (and other mammals), either by DNA microinjection or homologous recombination in ES cells, and the successful expression of the inserted genes within an organism provides new insights and opportunities for biomedical research. Thus, transgenic technology constitutes an increasingly important tool for studying the regulation and function of genes. Moreover, the different experimental strategies employing either gain-of-function or loss-of-function approaches allow the generation of specific animal models for human neurodegenerative diseases[61,62]. Detailed analysis of the underlying mechanisms will provide new insights into the genetic origin of these diseases, and will lead to a better understanding of normal and abnormal physiological processes. This knowledge will improve our diagnostic tools and offer new routes for therapeutic intervention.

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The **Review** section of *TIBTECH* is a platform for balanced and concise presentations of specific areas of research. If you would like to contribute, please contact the Editor.

Selective targeting of bioactive compounds to mitochondria

Michael P. Murphy

Mitochondrial function can be manipulated selectively by targeting bioactive compounds to mitochondria in living cells. Current targeting mechanisms involve harnessing either the mitochondrial membrane potential or the mitochondrial protein-import machinery to take up molecules linked to lipophilic cations or mitochondrial signal peptides, respectively. Compounds localized like this have been used in studies of mitochondrial metabolism and show potential as anticancer drugs. In addition, because mitochondria play a role in many critical cell processes, mitochondrial targeting has the potential to protect, repair or kill cells selectively, and may become a key tool in the development of gene therapy for mitochondrial DNA diseases.

Mitochondria play a major role in the metabolism of eukaryotic cells[1] by making significant contributions to the synthesis of ATP and the regulation of cytosolic calcium homeostasis and of cell death (Box 1). Consequently, mitochondrial dysfunction contributes to human disease and ageing[2]. It is possible to kill cells selectively, or alternatively to prevent their death, through the selective manipulation of mitochondrial function. One way to achieve this goal is to target bioactive compounds to mitochondria. Recent studies have established that compounds can be targeted to mitochondria using strategies that exploit two unique properties of the organelle: the large membrane potential across the mitochondrial inner membrane (Box 2) and the mitochondrial protein-import machinery (Box 3). Here I review methods of targeting molecules to mitochondria and discuss how this approach might be exploited to design novel drugs and therapeutic strategies of widespread applicability.

Mitochondrial accumulation of lipophilic cations

A substantial potential is maintained across the inner membrane of mitochondria. In isolated mitochondria, the maximum membrane potential is 180–200 mV, close to the largest voltage a lipid bilayer can sustain without dielectric breakdown[3]. In intact, living cells this membrane potential is somewhat lower (typically 120–170 mV), due to it being utilized for ATP synthesis. Importantly, the mitochondrial membrane potential is substantially larger than any other in cells, with the result that cations are selectively attracted to mitochondria. Polar cations require a specific carrier protein to cross the lipid bilayer of the mitochondrial inner membrane. In contrast, lipophilic cations pass easily through lipid bilayers and can be accumulated in the mitochondrial matrix, because their positive charge is screened or delocalized over a large surface area[4,5] (Box 2).

The Nernst equation (Box 2) describes the dependence of lipophilic-cation accumulation on the magnitude of the membrane potential. Significantly, every 61.5 mV increase in membrane potential causes a tenfold increase in cation concentration within mitochondria[4]. Accordingly, the mitochondrial membrane potential in living cells will drive cation uptake to give concentrations 100–1000 times higher than in the cytoplasm. The plasma membrane potential (30–60 mV) similarly drives the cytoplasmic accumulation of extracellular lipophilic cations. Together, the plasma and mitochondrial membrane potentials can account for the selective accumulation of lipophilic cations in mitochondria, provided that there is no active efflux pathway and that the cation is not metabolized or immediately toxic to the cell[4]. This property was used unwittingly over eighty years ago, by microscopists who found that the cationic dye Janus green selectively stained living mitochondria[6].

Two groups of lipophilic cations have been used widely to measure mitochondrial membrane potential and to visualize energized mitochondria (i.e. those with substantial membrane potentials) within cells. Phosphonium salts such as methyltriphenylphosphonium (Box 2) were shown by Skulachev and coworkers to be taken up rapidly in an energization-dependent manner by isolated mitochondria and

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PII: S0167-7799(97)01068-8

by mitochondria in living cells[7]. The distribution of the cation equilibrates according to the relative magnitudes of the mitochondrial and plasma membrane potentials and can be measured using radiolabelled compounds, enabling the mitochondrial membrane potential to be calculated from the Nernst equation (Box 2)[8]. Chen and co-workers[5] developed fluorescent lipophilic cations such as rhodamine 123 (Box 2) and JC-1, which are taken up by energized mitochondria within cells. These probes enabled mitochondria to be visualized within cells by fluorescence microscopy, and their energization to be quantitated by flow cytometry.

Lipophilic cations as anticancer drugs

Lipophilic cations have been evaluated as anticancer drugs because many carcinoma cells have a higher mitochondrial membrane potential than normal cells, leading to selective uptake[5]. Changes in plasma-membrane potential and lipid composition may also favour the selective accumulation of lipophilic cations by cancer cells[9,10]. Chen and colleagues have found many lipophilic cations that disrupt cell function and have selective activity against cancer cells both *in vitro* and *in vivo*[9,11]. Some of the most promising compounds are currently undergoing clinical trials[9,12].

How does the mitochondrial accumulation of lipophilic cations kill cells? High intramitochondrial concentrations of these cations increase the permeability of the mitochondrial inner membrane and inhibits mitochondrial enzymes nonspecifically, decreasing ATP production and ultimately leading to cell death[8]. Even relatively simple lipophilic cations such as tetraphenylphosphonium disrupt mitochondria by increasing the proton permeability of the inner membrane and inhibiting respiration[8]. The selective toxicity is evidently due to the higher concentrations of these cations in cancer cells[13–15]. Other lipophilic cations with anticancer activity inhibit the respiratory chain and F_0F_1-ATP synthase or disrupt transcription or translation in mitochondria[12,13,15]. While these more-specific actions contribute to the toxicity of these compounds, they are not particularly effective or specific mitochondrial inhibitors in comparison with classical inhibitors such as antimycin, oligomycin or myxothiazol.

Most untransformed cells have energized mitochondria and will therefore also be affected by approaches that rely on the mitochondrial membrane potential for selectivity. However, unlike many other cancer drugs, lipophilic cations do not act by damaging DNA and they are therefore less likely to lead to further mutation. Moreover, the toxic effects of these compounds are reversible[12]. The effectiveness of lipophilic cations against cancer cells can be increased by including some other treatment, such as exposure to laser radiation, hyperthermia or ionizing radiation[16,17]. Facilitating these treatments, mitochondria occupy a large proportion of the cell (5–20% of the cytoplasmic volume) and can act as an intracellular depot, holding large amounts of the compound, releasing it slowly to the rest of the cell[5]. Finally, it should be remembered that cells within solid tumours are often anaerobic, and consequently do not have a mitochondrial membrane potential generated by respiration (although reversal of the F_0F_1-ATP synthase may generate some membrane potential). Lipophilic cations may therefore be expected to be most effective against metastasizing cells or those on the periphery of tumours.

Using lipophilic cations to target passenger compounds to mitochondria

Lipophilic cations can be used as carriers to deliver bioactive passenger molecules to mitochondria (Box 2). For example, rhodamine 123 was used to direct the anticancer drug cisplatin selectively to cancer cells[16]. This approach was extended by Rideout and colleagues, who constructed a series of phosphonium salts incorporating protein-alkylating groups as passenger molecules, resulting in selective toxicity against cancer cells[18]. A more complex strategy involved the use of two different lipophilic-cation conjugates that reacted inside mitochondria to form a toxic product[19,20].

The use of specific mitochondrial inhibitors as passenger molecules may lead to more-effective cancer therapies. However, respiratory inhibitors may not be the most effective anticancer agents, unless their inhibition is essentially irreversible. This is due to the fact that the resultant loss of membrane potential would lead to rapid efflux of the inhibitor from the mitochondria and its redistribution to other cells. A better strategy may be to use inhibitors of the F_0F_1-ATP synthase, because inhibition of ATP synthesis would increase the membrane potential and thus the sequestration of inhibitor by affected cells, while gradually starving the cell of ATP.

Phosphonium salts can also be used to deliver passenger molecules intended to have more subtle effects on mitochondrial metabolism. For example, as mitochondria are a major source of free radicals, it might be expected that mitochondrial targeting of antioxidants would give protection against the oxidative stress associated with a number of human pathologies[2]. To this end, my group has developed a mitochondrially targeted thiol compound by linking it to a phosphonium carrier[21,22]; a broader range of mitochondrially targeted antioxidants is under development. These compounds are being used to investigate the pathological consequences for the cell of mitochondrial oxidative stress, but they also have the potential to be used therapeutically to protect mitochondria from oxidative damage.

Using the mitochondrial protein-import machinery to deliver macromolecules

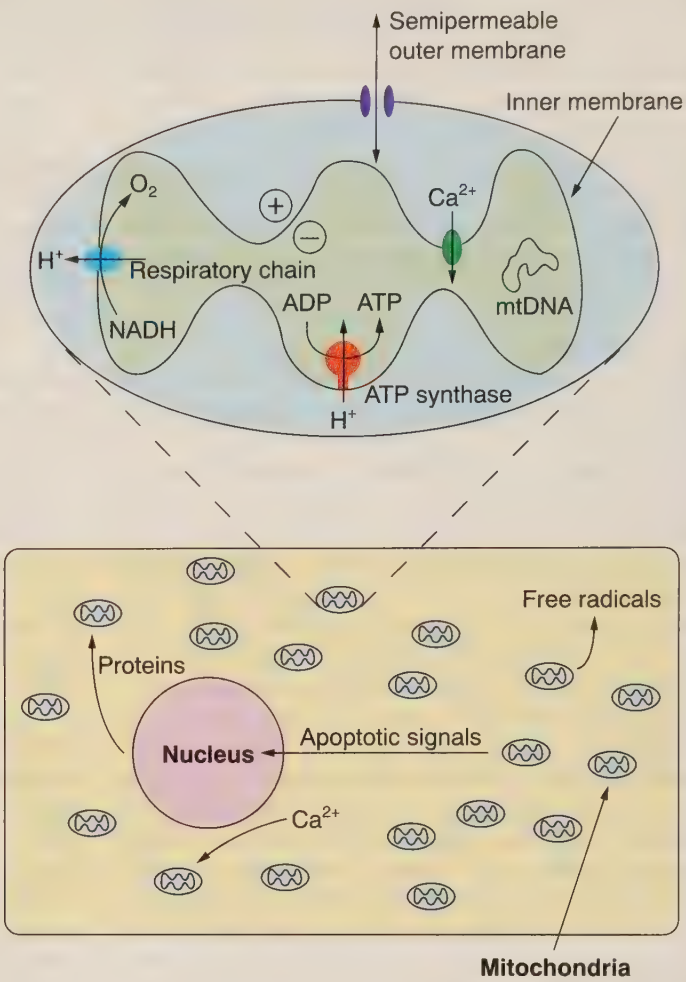
An amino-terminal peptide-targeting ('signal') sequence directs proteins to the mitochondrial matrix (Box 3). By fusing the mitochondrial signal sequence to the amino termini of nonmitochondrial proteins, the resulting chimera can be targeted to the mitochondrial matrix. For example, a fusion protein formed from the signal sequence of the mitochondrial protein cytochrome oxidase subunit IV and the

Box 1. Properties of mitochondria

Eukaryotic cells typically contain several hundred mitochondria, each enclosed by two membranes. A major function of mitochondria is to produce ATP by a process termed *oxidative phosphorylation*, in which the mitochondrial respiratory chain uses oxygen to oxidize reduced electron carriers such as NADH, then conserves the resulting energy by pumping protons out of the mitochondrial matrix. The ensuing proton-electrochemical gradient across the mitochondrial inner membrane comprises a pH gradient (basic inside) and a membrane potential (negative inside). The energy stored in this electrochemical gradient is used in the synthesis of ATP, by letting protons flow back through the F_0F_1-ATP synthase into the mitochondrial matrix. ATP leaves the mitochondria via the adenine-nucleotide carrier and diffuses out to the cell through the semipermeable outer membrane.

Most mitochondrial proteins are encoded by nuclear genes, assembled on cytoplasmic ribosomes and imported into mitochondria post-translationally (Box 2). However, some mitochondrial polypeptides (13 in mammals) are encoded by the mitochondrial DNA and are assembled on mitochondrial ribosomes. In humans, there are typically 2–10 mitochondrial DNA molecules of 16 569 bp each per mitochondrion. Mutations to mitochondrial DNA lead to a number of debilitating human diseases that are generally characterized by progressive neuromuscular and cognitive dysfunction[30].

The mitochondrial respiratory chain is a major source of free radicals in cells[36]. Electrons leak from electron carriers and react with oxygen to form the damaging radical superoxide. About 1–4% of the oxygen we breathe is converted to superoxide in this way[36], and mitochondria are consequently continually exposed to high levels of oxidative stress. Elevated mitochondrial oxidative stress is thought to contribute to a variety of degenerative diseases[2]. Mitochondria can sequester excess cytosolic calcium[37], a process that appears to be important in protecting cells against damage. Conversely, disruption of mitochondrial calcium homeostasis can lead to cell death. Mitochondria also play a regulatory role in apoptosis (programmed cell death) by releasing signalling molecules that activate the programmed-cell-death pathways throughout the cell[38].

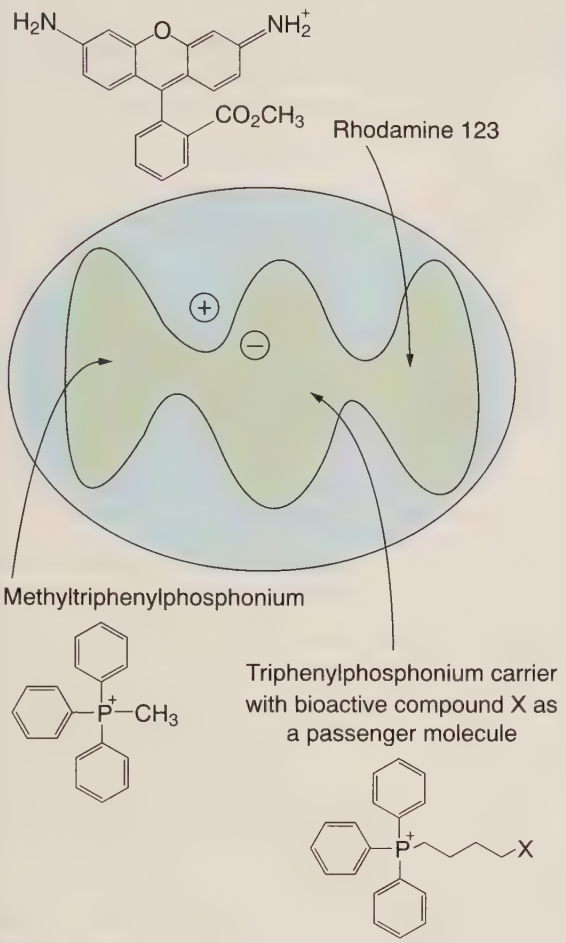


cytosolic enzyme dihydrofolate reductase was successfully imported into isolated mitochondria[23]. Transfection of yeast[24] or mammalian[25] cells with DNA encoding similar chimeric fusion proteins led to mitochondrial localization of active enzymes. This strategy has also been used to target the calcium-sensitive protein aequorin to mitochondria[26] (allowing the measurement of mitochondrial calcium levels within intact cells) and to construct a mitochondrially targeted version of Green Fluorescent Protein (allowing the visualization of mitochondria within living cells)[27].

These delivery mechanisms may also be useful for mitochondrial gene therapy[28]. Mutations to nuclear-encoded mitochondrial genes can, in principle, be repaired by transfecting cells with the corrected gene, the product of which will be naturally targeted to the mitochondria[29]. However, many mitochondrial genetic diseases are due to mutations of mitochondrial DNA[30] (Box 1), making gene therapy more problematical. Strategies using the mitochondrial protein-import machinery show promise – for example, one possibility is to introduce into the cytoplasm a corrected version of the defective mitochondrial gene, modified to incorporate a mitochondrial signal sequence and with appropriate changes in genetic code and codon usage[28]. In yeast, cytoplasmic expression of the mitochondrial ATPase-8 gene restored F_0F_1-ATP-synthase activity to yeast that lacked this gene[31]. Human gene therapies for diseases associated with point mutations to mitochondrial polypeptide genes might be developed from this approach, although complications could arise from the continued expression of the defective polypeptide, such as preventing incorporation of the corrected polypeptide into the final complex[28]. Furthermore, the cytoplasmic expression of mitochondrially encoded polypeptides might damage the cell.

Most mitochondrial DNA diseases are due to mutations of tRNAs, or to large deletions from mitochondrial DNA. To develop gene therapies for these diseases, it will be necessary to deliver appropriate

Box 2. Accumulation of lipophilic cations in mitochondria

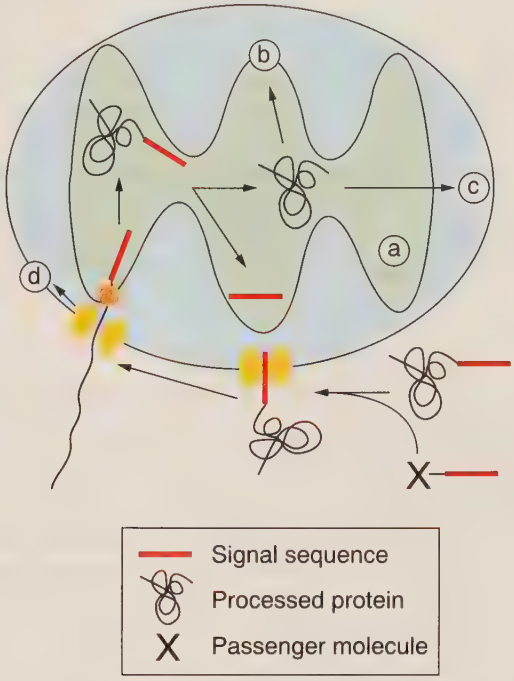


Mitochondria accumulate lipophilic cations, such as methyltriphenylphosphonium, rhodamine 123 and the derivative of triphenylphosphonium attached to a bioactive passenger molecule (X), because of the large membrane potential across their inner membrane. The distribution of cations in equilibrium with the membrane potential is described by the Nernst equation, subject to membrane-binding corrections[8]:

$$\text{membrane potential (mV)} = 61.5 \log_{10} (C_i/C_o)$$

where C_i is the concentration of cations inside the mitochondrion, and C_o is the concentration of cations outside.

Box 3. Protein import into mitochondria



Most mitochondrial proteins are imported into the organelle following assembly on cytoplasmic ribosomes. Protein import is directed by a signal sequence that generally comprises an amino-terminal amphipathic helix of 20–30 amino-acid residues that targets the protein to the mitochondrial matrix (a). Additional internal sequence motifs can act as sorting signals, often by interrupting protein translocation across the membrane, and lead to subsequent sublocalization to the mitochondrial inner membrane (b), intermembrane space (c) or outer membrane (d). Several other pathways also exist to direct proteins to each mitochondrial subcompartment, and this chain of events is not exclusive or universal[39,40]. The signal peptide is usually removed from the protein after its import. Before import, the cytoplasmic protein is maintained in an unfolded state by chaperonins, allowing the amino-terminal signal sequence to bind receptors on the mitochondrial outer membrane. The protein is then passed through a transmembrane pore, enabling insertion into the inner membrane by a process that requires a membrane potential. Subsequently, a transmembrane pore forms, enabling the protein to be threaded through to the matrix. Proteins, peptides or DNA (indicated by X above) can be targeted to the mitochondrial matrix in cells by linking them to signal peptides.

nucleic acid sequences directly to mitochondria. While mitochondria do have RNA-uptake systems[32], they are poorly understood in mammals. The most effective strategy at present is to modify a particular nucleic acid sequence so that it is imported by the mitochondrial protein-import machinery. Oligonucleotides coupled to mitochondrial peptide signal sequences are taken up by mitochondria[33,34]. One possible strategy is to introduce a corrected mitochondrial DNA sequence into the organelle, but a more promising approach is to block the replication of defective genes and thus enable the cell to become repopulated with undamaged mitochondrial DNA. This is possible because, even though a high proportion of each cell's thousands of mitochondrial DNA molecules might be mutated[30], the remainder are undamaged (a condition termed heteroplasmy). Therefore, if the replication of mutated mitochondrial DNA is selectively blocked, the remaining wild-type molecules may restock the cell, suppressing or eliminating the disease phenotype. Recent work

by Lightowlers and co-workers[35] indicated that this strategy works *in vitro* and that peptide nucleic acid (PNA) oligomers are more effective than oligonucleotides themselves, due to their increased stability and better DNA-binding characteristics [see D. Corey, *TIBTECH* June 1997]. They showed that appropriate PNA oligomers could bind to mutated mitochondrial DNA sequences and block their replication, without affecting normal DNA sequences[35]. Delivery of signal-peptide–PNA conjugates to the mitochondria of living cells is an attractive candidate for developing effective gene therapies for mitochondrial DNA diseases[35].

Future perspectives

The targeting of bioactive compounds to mitochondria within living cells is feasible, and has already been used to elucidate mitochondrial function and to design candidate anticancer drugs. The potential clearly exists for more-effective anticancer drugs that, for example, use lipophilic cations to deliver more-specific and -potent mitochondrial inhibitors. New classes of drugs may evolve from compounds designed to modulate specific mitochondrial functions in subtle ways, because mitochondrial dysfunction contributes to cell damage and death in several ways (Box 1). For example, compounds designed to increase the mitochondrial capacity for calcium or to protect against oxidative stress could prevent mitochondrial damage. Such compounds might also be effective in slowing the development of degenerative disorders that involve mitochondrial damage[2] (e.g. Parkinson's disease). It also might be possible to construct compounds that selectively induce cell death in some classes of cells, or prevent it in others, given the important signalling role of mitochondria in apoptotic cell death.

Mitochondrial targeting also has considerable potential for mitochondrial gene therapy. Mitochondrial DNA diseases are relatively easy to detect, often lead to progressive deterioration and currently lack an effective treatment, making them good candidates for gene-based interventions. Progress in application of the mitochondrial targeting approaches described here will depend on the development of effective means to introduce bioactive compounds into living cells within a patient. In future work, it will be interesting to see if lipophilic cations can facilitate the delivery of larger molecules such as peptides, oligonucleotides or peptide nucleic acids to mitochondria within cells. Novel ways to modify mitochondrial metabolism, for example by targeting nonmitochondrial enzymes to disrupt or counteract the effects of mitochondrial gene defects, might also be explored. Similarly, mitochondrially targeted metabolites (e.g. quinones linked to phosphonium cations) could be used to overcome metabolic blocks arising from mitochondrial gene defects. The design of mitochondria-specific bioactive compounds is in its infancy, but considerable potential exists here for the development of new classes of mitochondrial drugs and therapies.

Acknowledgements

I thank Rob Smith, Rhys Burns and Bob Lightowlers for many helpful discussions on these topics. I am grateful to Mike Hubbard and Herman Pel for their helpful comments on the manuscript. Work in the author's laboratory is supported by grants from the Health Research Council of New Zealand and the Neurological Foundation of New Zealand.

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Where genetics meets medicine

Molecular Diagnostics for the Clinical Laboratorian

edited by W. B. Coleman and G. J. Tsongalis, Humana Press, 1997. US$79.50 (x + 390 pages) ISBN 0 89603 373 2

This book endeavours to cover molecular techniques and their application in the clinical laboratory. It is divided into four parts: basic molecular biology, molecular technologies, applications to molecular pathology and issues for the clinical molecular pathology laboratory.

Introductory chapters are often overlooked by the reader, especially if only dipping into a book, but 'A historical perspective on the clinical diagnostic laboratory' makes interesting reading if your history of medicine is at all rusty. The remainder of Part I covers the basic structure, function and isolation of nucleic acids, and outlines the molecular techniques involved in cloning and sequencing. It is by no means comprehensive, but provides a concise and sound introduction to the subsequent parts of the book.

Part II of the book gives a reasonably in-depth consideration of Southern and northern blotting, PCR, RT-PCR, and *in situ* hybridization. Some topics, such as precautions to minimize RNase contamination, are duplicated between chapters. This does, however, enable each chapter to be read as a self-contained unit. A few protocols are included but, while laboratory scientists may pick up some useful tips, this book is not primarily a protocols manual. There is a well-written overview of PCR-based methods for detection of known mutations and for mutation scanning. However, the inclusion of a chapter on gene therapy feels a little out of place in a book otherwise devoted to clinical molecular diagnostics.

A wide-ranging overview of genetic disorders is undertaken in the third section, including neurological and neuromuscular disorders, endocrine disorders, cardiovascular disease, and neoplasia. The emphasis is more clinical, but the language is easily accessible to non-clinicians. Individual diseases are, by necessity, only briefly summarized but these chapters provide a broad snapshot of what molecular diagnostics can currently offer. The concluding chapters take a brief look at quality control issues (an important topic that perhaps deserved more emphasis), ethics and future prospects.

Overall, the book is well written and successfully integrates clinical and molecular-genetic information. The coverage of basic molecular techniques and their scope and limitations will appeal to the increasing number of clinicians who need to embrace molecular-genetic technology. Students and trainee scientists will also find it a useful text, whereas an experienced clinical molecular scientist will probably have most of the information already available to them elsewhere. As recognized in the conclusion, the field of molecular genetics continues to progress at a great pace and the biggest difficulty with any book on this topic is the speed with which it will inevitably date.

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Better alfalfa

Biotechnology and the Improvement of Forage Legumes

edited by B. D. McKersie and D. C. W. Brown, CAB International, 1996. UK£75.00 hbk (464 pages) ISBN 0 85199 109 2

Although there is a discrepancy between the contents and the title of this book, I believe this should not deter the reader. The title promises biotechnology in relation to forage legumes, but delivers mostly on one forage legume, namely alfalfa. It attempts to show how biotechnology is being applied, with other breeding techniques, to obtain a better understanding of the ways in which alfalfa responds to stresses imposed by pests, diseases, drought, low temperature, salt and soil acidification. There are also additional chapters dealing with other topics including: the manipulation of condensed tannin accumulation in order to confer bloat protection, enhance bypass protein levels or increase palatability and digestibility; signalling compounds involved in plant–microbe interactions, with special emphasis on the interaction of *Rhizobium* with alfalfa roots; and the potential of genetically engineered alfalfa to act as a bioreactor. The 17 chapters are contributed by a range of experts from around the world, and the contents are generally well edited, cross-referenced and well integrated for a multiauthored text.

Compared with food and fibre plants, the species of the pastoral zones of the world have received very little biotechnological attention. However, it is clear from this book that alfalfa is amenable to genetic transformation and that there are a number of ways in which this highly desirable forage plant might be improved further using recombinant and other DNA-based techniques. While this book was in preparation and in press, there have been reports of the genetic modification of many other forage legumes, and these advances pave the way for the manipulation of genes capable of enhancing both the agronomic performance and the feed value of these plants for the animal-based industries that rely heavily on high quality, and thus legume enriched, pastures for maximum production.

I recommend this book as an introduction to the emerging area of the application of biotechnology to forage legumes. It is suitable for researchers and for advanced students on applied biology courses. The editors and the many authors are to be congratulated on the balanced integration of the new technology with existing proven methods of plant improvement.

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The mystery of the endothelium

Endothelium-derived Hyperpolarising Factor

edited by P. M. Vanhoutte, Harwood Academic Publishers, 1996. UK£69.00 (338 pages) ISBN 3 7186 5929 8

The endothelium plays an important role in maintaining vascular homeostasis by releasing several vasodilators, including prostacyclin, nitric oxide and endothelium-derived hyperpolarising factor (EDHF). Although the pathophysiological roles of the first two substances have been extensively investigated in the past two decades, those of EDHF still remain a mystery in our understanding of the endothelial control of vascular function.

Endothelium-derived Hyperpolarising Factor, edited by Dr Vanhoutte, consists of the proceedings of the first international symposium devoted to this factor, which was held in France in 1995. The book, with 37 chapters, summarizes for the first time the latest knowledge on all aspects of EDHF. Indeed, it is truly an international book, with contributions from workers in various fields and from all around the world.

Endothelium-derived Hyperpolarising Factor consists of four series of chapters. The first series (Chapters 1–8) deals with evidence for the existence of EDHF and its contribution to vascular responses. Here, convincing evidence for a hyperpolarising factor other than prostacyclin or nitric oxide is presented. The second part (Chapters 9–21) focuses on the nature of EDHF and the mechanisms underlying its release. In particular, cytochrome P450 monooxygenase metabolites have been extensively discussed as possible candidates for EDHF. The third set of chapters (Chapters 22–27) discusses the effects of EDHF on vascular smooth muscle cells and its potential contribution to the pathophysiology of the vascular wall. Here, the importance of EDHF in microvessels is especially emphasized. The final part of the book (Chapters 28–37) deals with the alterations in the release and/or action of EDHF in various animal models of vascular diseases and its possible roles in human physiology and pathophysiology. Indeed, it may be the case that EDHF-mediated hyperpolarization and relaxation are reduced under various pathological conditions in animals (e.g. aging, hypertension, hypercholesterolaemia and diabetes mellitus), and this could also be the case in humans.

The editor has done a fine job in producing a multiauthored text with a high degree of internal consistency in terms of style and content. It also should be pointed out that all the figures presented in the book are comprehensible and consistent as they have been produced by a single illustrator, who is fully familiar with cardiovascular research. The list of references at the end of the book contains almost all the currently available information on EDHF.

Endothelium-derived Hyperpolarising Factor will be of interest to a wide variety of readers, including not only physiologists, pharmacologists and undergraduate and graduate students who are interested in vascular biology, but also clinical researchers and physicians who treat patients with cardiovascular diseases.

In conclusion, I can recommend this book to a wide variety of readers, as it is a valuable source of the latest information on EDHF.

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Practical protocols for neuropeptides

Methods in Molecular Biology, Vol. 73: Neuropeptide Protocols

edited by G. B. Irvine and C. H. Williams, Humana Press, 1997. US$69.50 (384 pages) ISBN 0 89603 399 6

There is a steady flow of handbooks dealing with various aspects of neuropeptide and neurohormonal peptide research methodology. This creates a special problem for the potential consumer: when to make up your mind to buy? In some ways, the decision-making reminds me of the way I feel when about to buy a computer. Should I wait for the latest model to appear on the market, with all the latest technology and gadgets and thereby (hopefully) better meeting my demands?

The approach taken by *Neuropeptide Protocols* is a practical one, and each chapter contains notes about potential pitfalls and tips on how to avoid failures. On the whole this is an advantage, but the turnover of techniques within the field of neuropeptide research is high and details in the different protocols change quite frequently, and consequently the survival time of a detailed description is rather short. Further, some of the methods described, for example the MALDI-TOF-MS (Chapter 14), seem exotic to the extent that it is questionable whether they have a place in a volume meant for a broad audience. Also, many chapters have surprisingly few references.

That said, *Neuropeptide Protocols* is a comprehensive volume, authoritatively covering most, but not all, of the techniques that can possibly be used in neuropeptide research. Examples of the gaps in this volume include the lack of a chapter on *in situ* hybridization (even though this is a versatile, powerful and now quite frequently used tool for identifying the cellular site of neuropeptide synthesis) and of information on the related subject of cellular quantification of an mRNA signal by computerized image analysis (a technique permitting studies on the regulation of neuropeptide gene expression). Another problem inherent in a broad-spectrum volume of this kind is that very few laboratories are capable of using more than a fraction of the methods described. Consequently, a major part of the content is probably left unread.

All in all, if you are a neuropeptide chemist, and particularly if you are about to get started at the bench, you may find this volume valuable. If you are in other fields of neuropeptide research, you might be better waiting for the next one, which may better suit your purposes, and that may be just around the corner.

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Racemic temperature and stereochemistry

Letter

Dear Sir,
This is a discussion of the concept 'racemic temperature' and its application to stereochemistry proposed in a previous paper in this journal[1]. We hope that through this discussion, a better understanding of this temperature effect can be reached.

Thermodynamically speaking, the following relationships hold:

$$\Delta\Delta G^{\ddagger} = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger} \qquad (1)$$

where $\Delta\Delta G^{\ddagger}$, $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ are the differences in the free energy of activation, the enthalpy of the activation and the entropy of the activation between the two isomers involved in the reaction and T is the temperature; and

$$-RT \ln E = \Delta\Delta G^{\ddagger} \qquad (2)$$

where R is the molar gas constant and E is the enantiospecificity ratio. When E is equal to 1, that is, the selectivity of the enzyme to the two isomers is identical, the product will be a racemate; in this case, when $\Delta\Delta G^{\ddagger} = 0$ in the above equation, the temperature is called the 'racemic temperature', T_r. Thus, from Eqn 1

$$T_r = \Delta\Delta H^{\ddagger} / \Delta\Delta S^{\ddagger} \qquad (3)$$

Accordingly, it has been asserted that when reaction temperature, T, is less than T_r, the stereochemical process of the reaction is dominated by the activation-enthalpy difference, and the stereochemical purity of products will be expected to decrease with increasing temperature[1]. However, when the temperature changes, $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$, also change, as these are functions of temperature. In theory, there may exist a T_r that is specific to a particular system, but, to date, this has not been possible to predict.

Also, the method given in a previous paper for obtaining $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ is questionable[2]. In that case, $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ were regarded as constants and were obtained as the slope and intercept of the line for the linear relationship between $RT \ln E$ and T. In practice, it is only a hypothesis that $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ are constants. As T_r was raised, it was compared with the 'isokinetic temperature'[3], but these two concepts may not be similar. Furthermore, one cannot judge the existence of T_r unless it is found that there is an inversion of stereoselectivity in the experiment. To the best of our knowledge, no other group has established a value for T_r.

Above all, if $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ are not constant, mathematically speaking, there may be more than one value for T_r. The experimental results of Parmar[4], which showed that there are two inversions of enantioselectivity with changes in temperature, may support this hypothesis. T_r is a term relevant to a narrow temperature change of biocatalysis that could not be predicted theoretically. It is not, therefore, easily applied, as the previous author suggested[1].

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Response

Reply

Dear Sir,
G. Duan and J. Y. Chen have raised some interesting concerns regarding my treatment of the effects of temperature on the stereochemistry of enzymatic reactions, published in *TIBTECH* last year. I am an experimentalist, not a theoretician, and the mathematical treatment given in the paper was developed out of the necessity of explaining our results after studying the effects of temperature on the stereospecificity of thermostable secondary alcohol dehydrogenase[1]. If Duan and Chen can show me a better method to analyse these data, I will gladly adopt it.

Their main criticism of the mathematical treatment deals with the constancy with varting temperature of the differential Eyring parameters, $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$. Because the equation presented in the paper is derived from the well-known Eyring equation, which in turn is related to the classical Arrhenius equations that describe the effects of temperature on reaction rates, it might be instructive first to consider the situation in the absence of stereoselectivity. It is customary to obtain the Eyring parameters, $\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$, for enzymatic reactions by plotting $\ln k$ against $1/T$, a graph whose slope will provide $\Delta H^{\ddagger}/R$ and whose intercept will be $\Delta S^{\ddagger}/R$. The equation given in the paper is simply a transformation of the Eyring equation, and has a slope of $\Delta S^{\ddagger}$ and an intercept of $\Delta H^{\ddagger}$. Regardless of the methods of plotting the data, identical values for the parameters are obtained.

Given the restricted range of temperatures available for performing enzymic reactions, generally about 50°C and not often more than 80°C, even in the case of thermophilic proteins, linear behavior in such plots is the rule, not the exception, suggesting that the parameters are approximately constant. Nonlinear behavior in Arrhenius or Eyring plots, when seen, is usually seen as a 'break' between two linear regions, and is often interpreted in terms of changes in the protein structure (e.g. a conformational change) with temperature. Now, when we are dealing with stereochemistry, $\Delta\Delta H^{\ddagger}$ (or $\Delta\Delta S^{\ddagger}$) is likely to be the difference in the slopes (or intercepts) of two linear equations in T, and thus constant.

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Even if the individual plots are nonlinear, if the nonlinearity is present equally in both of the curves, due to conformational changes that affect the reaction of both substrates equally, their difference may still be linear. The method given for determining the apparent differential Eyring parameters is thus reasonable if the data are linear, as ours are. However, the magnitudes of these parameters are likely to be sensitive to other physical variables, such as pH, buffer structure, cofactors, cosolvents, etc., and so they must be obtained and compared under carefully controlled conditions.

Nonetheless, I recognise that k_{cat}/K_m for an enzymatic reaction is not an intrinsic constant, but a complex mixture of intrinsic kinetic constants related to both binding and chemical processes, which may differ in their individual activation parameters, and it is also possible that the rate-limiting step may change with temperature. If so, then a 'turn-around' in the effect of T on *E*, as reported by Parmar *et al.*[2], may well occur. Examples of this behavior in stereoselective nonenzymatic reactions have been documented by Scharf and co-workers[3]. However, given the limited number of temperature data points and the lack of statistics of the measurements in the work of Parmar *et al.*[2], it is premature to conclude that these results are an enzymatic example of this principle.

Finally, Duan and Chen state that no one else has published T_r for an enzyme reaction. This may be true at the present time, but I am aware of other groups that have recently performed similar measurements, and I am certain that other cases of T_r measurements for enzymes will be forthcoming in the near future. However, 'isoenantioselective temperatures' have been published for the separation of enantiomeric compounds by gas chromatography on chiral stationary phases[4,5]. These systems show a reversal of elution order with increasing temperature, and the data were analysed by the use of a thermodynamic equation analogous to the equation given in my article.

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Meeting report

Target practice – novel approaches to antimicrobial chemotherapy

Much of the development of biotechnology has benefited greatly from the historical preeminence of microbially produced products (and their derivatives) that possess antimicrobial activity, but it is now generally recognized that bacterial resistance to these molecules is widespread. Thus, many classes of organism, such as methicillin-resistant *Staphyococcus aureus* (MRSA) and vancomycin-resistant enterococci, currently give enormous cause for concern in clinical settings, especially following the demonstration of the transfer of vancomycin resistance from enterococci to staphylococci. One reason for the emergence of such resistance is that the number of primary pharmacological targets actually exploited by these molecules is exceptionally small, and, driven in part by the genomics revolution, it is now widely recognized that the next generations of antibiotics should be aimed at new and different ones. This was the theme of a meeting held recently at the Institut Pasteur*.

**New targets for new antibiotics*, organized by Jozsef Aszodi and Patrice Courvalin, Institut Pasteur, Paris, France, 4–6 June 1997.

Of the clinically useful antibiotics, approximately 55% are β-lactams, with macrolides and fluoroquinolones each comprising about 15% of the market, yet of possible new chemical entities only oxazolidinones are currently known to be in even phase-I clinical trials (Jozsef Aszodi, Hoechst Marion Roussel Research Centre, Romainville, France). Much of the problem of how to make progress follows from our general ignorance of how bacteria work – for instance, the genome of *Escherichia coli* strain O157 is 25% larger than that of strain K12, so simple subtractive methods of gene expression will rarely give clean answers; in addition, recognizing a good target is only the very first step to obtaining a good pharmacological agent (Julian Davies, University of British Columbia, Vancouver, Canada).

By definition, a good antibiotic target is one that is necessary for the growth of the target cell under most or all conditions, including *in vivo*, is typically found in most common pathogens (to give a broad spectrum of activity) and yet is not present in the human host. The bacterial cell-division process provides a number of such targets, including the ftsZ ring (Joe Lutkenhaus, University of Kansas, Kansas City, KS, USA) and Min proteins (Jean-Pierre Bouché, CNRS, Toulouse, France) as do a variety of steps in peptidoglycan biosynthesis (Jean van Heijenoort, Université Paris Sud, Orsay, France); 2D zymograms of peptidoglycan hydrolases coupled to MALDI-TOF mass spectrometry have identified new targets, including skp (Wolfgang Keck, Hoffman–La Roche, Basel, Switzerland).

According to one view, that of 'rational drug design', the availability of a 3D crystallographic structure can make the process of drug design very much easier. The structure of elongation factor EF-Tu, which is involved in protein synthesis, is now available. This protein is known to have 60–80% conserved homology among bacteria but only 30% homology to the equivalent eukaryotic EF-1a, and, of a number of structurally distinct natural products that bind and block its activity (kirromycin, pulvomycin, MDL62,879), none is toxic to eukaryotes. EF-Tu is extremely abundant in *E. coli* and, although the above molecules are unsuitable for reasons of pharmacokinetics, their antimicrobial activity suggests that EF-Tu could indeed be an excellent target (Khalid Islam, Hoechst Marion Roussel Research Centre, Romainville, France).

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PII: S0167-7799(97)01078-0

Much of the growth, development and response of bacteria to a changing environment is mediated by the σ factors. These control which promoters will be read by RNA polymerase and, while drugs capable to binding to the interface between such a protein and nucleic acids are likely to be generally cytotoxic, the interface between the σ factors and the core enzyme presents an attractive target (Abraham Sonensheim, Tufts University, Boston, MA, USA).

During protein synthesis, premature dissociation of peptidyl tRNAs can lead to their cytotoxic accumulation; they are normally recycled by a peptidyl-tRNA hydrolase, and substances that prevent its activity are a potential source of antibacterial compounds (Sylvain Blanquet, École Polytechnique, Palaiseau, France). Bacterial proteins are initially formed with an N-terminal N-formyl methionine and, while the methionine group is usually removed in both prokaryotes and eukaryotes, the removal of the formyl group by a specific deformylase is specific to bacteria (Didier Mazel, Institut Pasteur, Paris, France). The overexpression (and consequent specific inhibition) of this enzyme can be assayed by the ability to grow on N-formyl-methionyl leucine as sole source of these amino acids.

While the cell wall of bacteria has long been recognized (*a posteriori*) as a suitable antibiotic target because of its unique chemistry, it is evident that not only its construction but also the secretory supply of its building blocks can serve this purpose. Of the three known secretory pathways, a general secretory pathway (GSP) is common to both Gram-positive and Gram-negative bacteria (Tony Pugsley, Institut Pasteur, Paris, France), as are ATP-binding cassette-type transport systems (Cécile Wandersman, Institut Pasteur, Paris, France). As with other cell-surface targets, the GSP is especially attractive as it avoids potential permeability problems and those due to efflux pumps that must be negotiated by molecules whose targets are cytoplasmic; secA may be particularly suitable. The type-III secretory pathway is involved in injecting bacterial proteins into foreign hosts, and is essentially unique to invasive Gram-negative bacteria such as *Yersinia* spp.; such a system can, of course, represent an antibacterial target, but might also be subverted for the intracellular delivery of vaccines and other therapeutic proteins (Guy Cornelis, Université Catholique de Louvain, Brussels, Belgium).

The challenge of genomics is not the acquisition of the sequences *per se*, as the entire human genome could be sequenced if every inhabitant of China sequenced just three bases (perhaps, for safety, on each strand) (Sydney Brenner, Institute of Molecular Sciences, La Jolla, CA, USA), but for the bioinformatics to keep up with the flood of genomic data to enable it to be exploited to the full. Even the few (<10) whole microbial genomes (including baker's yeast) in the public domain have caused a reevaluation of phylogeny on a grand scale, and indicate that the 'minimal gene complement' of bacteria under the most favourable conditions (and thus, at the most simplistic level, the possible number of possible antibiotic targets) is some 250–300 (Eugene Koonin, National Center for Biotechnology Information, Bethesda, MD, USA). Horizontal gene transfer on a large scale greatly complicates the rRNA-based 'tree of life', in which the Archaea had been seen as inclined towards the eukaryotic branch. Automated tools now allow the reconstruction of metabolic pathways in organisms as they are sequenced (Evgeni Sel'kov, European Bioinformatics Institute, Cambridge, UK). As genomics leads to functional genomics, and we move swiftly to the post-genomic era, the flood of data will increase even more. The big pharmaceutical houses are already exploiting oligonucleotide chip arrays and 2D electrophoretic analyses of the proteome to assess the expression of the ribonucleome under different conditions (Chris Gray, Hoffman–La Roche, Basel, Switzerland). The data emerging from the mycobacterial genome sequencing projects (and, to the great credit of those involved, being made available on the Internet as soon as they are checked) have already produced many surprises in *Mycobacterium tuberculosis*, such as a series of polyketide synthases (and, consequently, many new targets for drugs and vaccines) and the basis for the problematic multidrug resistance in this organism, seen in the array of β-lactamases, drug efflux pumps, etc. that its genome can encode. Indeed, in contrast to *Mycobacterium leprae*, which is apparently on the way to being eradicated, deaths from *M. tuberculosis* are greater than ever, and specific narrow-spectrum drugs must fill the 20-year gap before new classes of vaccine might become widely available (Stewart Cole, Institut Pasteur, Paris, France).

The problem of MRSA is so acute that an economic (as well as moral and scientific) case can be made for the development of drugs specifically to bypass methicillin resistance in such strains. Although its basis is the production of a novel penicillin-binding protein PBP 2′, a number of other factors essential for methicillin resistance (fem factors) are involved in the production of unique pentaglycyl groups in the polymeric staphylococcal cell wall, providing a clear and immediate target (Harald Labischinski, Bayer AG, Wuppertal, Germany). The sorting of surface proteins in such Gram-positive bacteria involves transpeptidases, which could also provide good targets (Olaf Schneewind, UCLA School of Medicine, Los Angeles, CA, USA).

The basis of vancomycin resistance in enterococci is now fairly well understood at the molecular level, and mainly involves the synthesis of D-alanine–D-lactate linkages instead of the D-alanine–D-alanine intermediates of peptidoglycan biosynthesis to which glycopeptides such as vancomycin normally bind (Peter Reynolds, Cambridge University, UK); a single phe ↔ tyr change is sufficient reversibly to effect resistance.

Multidrug resistance via efflux pumps is a well-recognized problem in both prokaryotes and eukaryotes (where the P-glycoprotein can mediate the resistance of certain tumours to cytotoxic drugs), and the inclusion of inhibitors of such pumps in antimicrobial cocktails could clearly play an important role in overcoming resistance (Olga Lomovskaya, Microcide Pharmaceuticals, Mountain View, CA, USA). However, it is necessary to inhibit all such pumps simultaneously, and their lack of substrate specificity evidently causes difficulties in finding a suitable inhibitor that would be active at low concentrations.

As with human warfare, an important class of target involves the systems used by the 'enemy' to sense what is going on in their environment and to communicate this between themselves. In sensing, many of the environmental changes are detected and mediated via more-or-less-highly conserved '2-component' systems involving histidine kinases, which have no counterparts

in animals (Tom Silhavy, Princeton University, Princeton, USA); as *E. coli* has some 28 of these, resistance is unlikely to prove a problem. In communication between Gram-negative bacteria, an important general 'language' involves N-acyl homoserine lactones (Gordon Stewart, Nottingham University, UK); because these often induce their own synthesis, the environmental levels reflect the bacterial concentration, and can be used by the organisms (in 'quorum sensing') to determine when this is sufficient to make the expression of exoenzymes and other virulence factors profitable. Inhibiting their synthesis or activity could therefore block virulence. In fact, one such molecule (OdDHL) in *Pseudomonas aeruginosa*, an opportunistic pathogen, is itself a virulence factor, as it can inhibit the inflammatory response in which lipopolysaccharide (LPS) induces the formation of TNF-α in lymphocytes. Antimetabolites from red algae are already known.

Thus, given that bactericidal antibiotics induce autolysis, which can lead to septic shock, it is reasonable to conclude that blocking the expression of virulence genes alone might be sufficient for effective antibiosis *in vivo*, and negative selection for multiple virulence genes *in vivo* is possible via signature-tagged mutagenesis (David Holden, Royal Postgraduate Medical School, London, UK); gene knockouts at loci identified in this fashion could decrease the dose of *Salmonella typhimurium* required for lethality by five orders of magnitude!

Of course there is much continuing interest in the exploitation of microbial diversity, and, indeed, of combinatorial biology, for the synthesis of novel antimicrobials. Certain polyketide synthases are synthesized as huge polypeptides that fold to form the modules of a helical, dimeric, multienzyme complex that adds groups to the growing polyketide *seriatim* (Jim Staunton, University of Cambridge, UK); mixing and matching modules (or, for that matter, mutating them or changing their order) could provide a host of novel antimicrobials and other bioactive substances, and similar possibilities exist for nonribosomal polypeptide synthases (Valéry de Crécy-Lagard, Institut Pasteur, Paris, France).

Although the nominal targets of existing antibiotics are now known, it is becoming clear that the successful ones can bind to multiple targets simultaneously – there are at least a dozen penicillin-binding proteins, while fluoroquinolones can bind both to DNA gyrase and to topoisomerase IV. Thus, care may need to be taken if an individual target for a novel antimicrobial drug is sought, and the success of multidrug therapy in treating mycobacterial disease, and the presumed decrease in the likelihood of resistance emerging from such a strategy, both favour the multiple-warhead approach to antimicrobial chemotherapy. But one thing is clear – we are in urgent need of new targets for new antibiotics.

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Biotechnology for world food security

The Michigan State University project 'Agricultural Biotechnology for Sustainable Productivity' recently organised a conference at Asilomar, a location on the Pacific coast with a very special meaning to molecular biologists. This is where, in 1974, many scientists came together for the first time to think about the social implications of gene technology. This time, the topic was limited to applications of modern biology to crops growing in the tropical regions of the world. It is striking how, in 23 years, gene technology has become daily routine in plant breeding, while some applications considered useful at that time, such as transferring the ability to fix nitrogen to cereals, have remained pipedreams. About a third of the participants of the meeting were workers in agricultural research from less-developed countries (LDCs). The debates at the meeting went beyond the strict realm of science and touched on technology transfer and the consequences of modern plant breeding for LDCs.

The world food problems are daunting: the United Nations Food and Agricultural Organisation estimates that, today, 800 million people do not have enough to eat, while the world population is increasing by around 80 million each year, with most of this increase in LDCs. New plant varieties may make it possible to improve food security without using more farm land, water or other scarce resources. It must be stressed, however, that new technologies alone are not sufficient. Other key issues are good government, access to minicredits for buying seed and fertilizer, fair prices for farm products, efficient transport systems to move produce to consumers and better education (particularly for women). I will cite only some selected examples of how various techniques of modern biology are applied to improve crop plants.

Bananas as a staple food

Bananas are by no means grown only as a cash crop, as we, living in the North, may think. In fact, only 10% of the world production (total 50 million tons yr^{-1}) are exported, the rest being consumed locally. As Oscar Arias (Agrobiotecnología de Costa Rica, Alajuela, Costa Rica) reported, banana plants are very susceptible to diverse diseases and pests, and therefore traditionally require a heavy input of agrochemicals. These are costly, and may be dangerous to the workers handling them if they are not well trained. In the past ten years, methods have been developed and applied in Costa Rica to propagate bananas asexually in the laboratory and in more-or-less sterile glass houses, so that the young plants are free of pathogens and, when they are about 30 cm high, can then be transferred to the field in a healthy state. With this head start, the plants bear fruit in the field after only nine months and then again every four months for many years. Even so, two fungal diseases still cause very serious problems. In the laboratory, several methods of micropropagation and of callus or cell culture are used to help find less susceptible strains of banana.

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PII: S0167-7799(97)01073-1

Elsewhere in the world, efforts have been made to transform bananas[1]. This may lead to new, agronomically useful varieties, and possibly also to fruit that could be used as oral vaccines for humans, after the transfer of relevant genes from human pathogens[2].

Improved rice varieties

The three most productive crops on a global basis are wheat, rice and maize, with worldwide annual harvests of 550, 520 and 490 million tons, respectively. By far the most research on wheat and maize is performed by companies in industrialized countries, but there are some notable exceptions. Natasha Bohorova (International Maize and Wheat Improvement Center, Mexico D.F., Mexico) reported on the successful transformation of tropical varieties of wheat with genes coding for agronomically important properties. However, these studies are still at an early stage.

With rice, the situation is very different, in that the leading research institution is an international center in the tropics rather than a company in the North. The International Rice Research Institute (IRRI) in Manila has been at the forefront in developing new varieties of rice for more than 30 years. Gurdev Kush, head of plant breeding at IRRI, who himself grew up on a farm in India, gave an overview of the impressive results already achieved. Half of all rice grown worldwide is derived in part or in whole from the breeding efforts of IRRI. Thirty years ago, the average rice yield was 4 tons per hectare, whereas today it is 10 tons. The newest varieties, which are about to be released, yield 12 tons. Most of the rice-producing countries in Asia, where 90% of the world's rice is grown, have become self-sufficient in rice.

The varieties that caused this 'green revolution' were obtained by traditional breeding. In recent years, however, molecular markers have been used in the selection process, giving breeders additional selection criteria. The next generation of varieties is being developed using recombinant DNA techniques, based on the regeneration of protoplasts. The aims are to introduce resistance to viruses, fungal diseases, pests and eventually even drought. Salt-tolerant varieties would also be of interest, as would varieties with increased nutritional value. For example, a variety of rice rich in vitamin A has been developed in Zurich[3].

Much of the work at IRRI is being performed in close collaboration with a large number of research groups in industrialized countries. It is important to realize that farmers need varieties adapted to the local conditions, such as climate, soil, etc., and so the newly introduced traits need to be transferred from the original variety to local varieties. This involves traditional breeding and can take a long time. Several laboratories in Japan and the USA are in the process of jointly sequencing the complete rice genome. This sequence information will, when completed, be very helpful to rice breeders by providing more accurate markers and enabling them to develop new ways of combining desired traits.

Sweet potatoes and cassava for local consumption

These two root crops are largely consumed where they are grown, and are also reproduced locally, rather than new seed being purchased from abroad. Both crops are important for subsistence farmers, because the plants grow in poor soil and store large amounts of starch. As a consequence of their local reproduction, seed companies in the industrialized countries have not invested much in research into these crops. Maud Hinchee (Monsanto) and Florence Wambugu (Kenyan Agricultural Research Institute, Nairobi, Kenya) reported the development of a virus-resistant strain of sweet potato. A coat-protein gene of the sweet-potato feathery mottle virus was introduced, and several hundred independent transformants are presently being investigated in containment. It is hoped that some will be field tested in Kenya next year.

Cassava (also known as manioc or tapioca) is the main staple of many poor farmers in tropical Asia, Africa and Latin America and allows survival in times of drought, because the roots can be kept in the ground for many months without significant spoilage. Recently, four groups of researchers have found ways of transforming cassava, the first crucial step in the introduction of recombinant gene technology for the development of new varieties[4–7]. Ann Marie Thro [Cassava Biotechnology Network (CBN), Cali, Columbia] organises a multinational network that has brought over 600 researchers into close contact with each other and with groups of cassava farmers since 1988. With sponsorship of the Dutch government, CBN assesses the needs of populations, identifies scientific problems, encourages relevant research and coordinates interaction and exchange among these projects.

New raw materials for industry

Suan-Choo Cheah (Palm Oil Research Institute of Malaysia, Kuala Lumpur) reported that approximately 60% of the palm oil sold on the world market is from Malaysia. Consequently, it comes as no surprise that Malaysia is making great efforts to increase production efficiency, to find new markets for these products and to modify them in such a way that they can be used in novel applications. In 1970, micropropagation was introduced for reproduction and selection, and this led to new varieties whose yield is twice that of the original varieties. The Institute has also developed DNA tests for the rapid screening of new varieties, and it also offers these tests to private seed companies for use in their own breeding programs.

The projects of the Palm Oil Research Institute go even further, and are aimed at changing the composition of palm oil. Using recombinant DNA techniques, the researchers want to produce an oil with a high oleic-acid content by introducing or deleting genes coding for specific enzymes involved in fatty acid biosynthesis. These modified oils may then be used by the chemical industry as feed for synthetic processes, thus opening up new market niches outside the food industry.

At the Asilomar meeting, several additional crops were also discussed. For all of them, methods of modern biotechnology such as micropropagation, cell, callus or embryo culture and DNA markers have been employed. Very often, but not always, transformation has been used to introduce new genetic traits that could not be brought in by traditional breeding. There were reports from workers in several countries on their activities in agricultural biotechnology, including Egypt, Chile and Peru, but unfortunately not China, where transgenic crops are planted on vast acreages.

Regulatory issues

Industrialized countries have legally binding rules on how to apply modern biotechnology. These require the existence of national biosafety regulatory bodies, such as the US Food and Drug Administration, the

US Department of Agriculture and the US Environmental Protection Agency. Some, but not all, LDCs also have such organizations in place. The stated policy of many multinational companies is not to do any recombinant DNA work where such regulations are not in place, but local researchers have been known to do field trials before any rules had been set up, which does not increase public confidence. Several countries, including Kenya, are in the process of setting up such a regulatory framework. The conditions for undertaking field trials will vary from country to country and crop to crop, depending on factors including which close relatives of a crop are endemic in that particular country. This will in turn affect the discussion on gene transfer through pollination.

The deregulation of transgenic maize in Mexico raised a number of unique questions, in part because Central America is the origin of maize, which is thought to be derived from teosinte, a native plant of the region. Ariel Alvarez-Morales (Center of Advanced Studies, Irapuato, Mexico), who is a member of the Mexican National Biosafety Committee, reported that Mexico was initially reluctant to deregulate transgenic maize, but realized that its importation and reuse as seed could not be realistically prevented, because large tonnages of mixed harvests are imported from the USA. The committee decided instead to organize a follow-up study to determine if there was any significant gene flow and look for possible consequences. It is interesting to note that old Mexican varieties of maize (landraces) have survived for many years alongside imported high-yield varieties, without any obvious loss of landrace identity. It is important that in the discussion on regulatory and safety issues, use is made of the great wealth of experience gained from the behavior of traditionally developed crops.

The Convention on Biological Diversity requires the establishment of biosafety regulations, as pointed out by Robert Frederick of the US Environmental Protection Agency. These will focus specifically on the movement of genetically modified organisms across national frontiers. A binding text can be expected by 1998 and should protect the safety interests of the LDCs without curtailing research in biotechnology and inhibiting world trade of the resulting products. It would be deplorable if a convention intended to increase technology transfer to LDCs in fact blocked it.

Protection of intellectual property rights

According to the Trade-related Industrial Property Rights regulations drawn up by the World Trade Organization, all nations should, within a few years, have some form of intellectual property right (IPR). Crop plants should be legally protected, either through patents or through a specific plant-variety regulation. At present, there is still a great deal of variation in IPR systems. The least-developed countries have virtually no IPR regulations, while the emerging nations appear to be keen on enacting patent legislation, in order to attract investments from large companies that would otherwise be reluctant to set up production facilities. Lerson Tanasugarn (Chulalongkorn University, Bangkok, Thailand) pointed out that there are already many valid biotechnology patents in Thailand, including some on locally invented products or processes. While whole plants cannot be patented in that country, DNA sequences, vectors and products can, thereby giving better legal protection than a plant-variety act would.

Many research institutions in the public sector prefer not to take out patents. This is the case for IRRI in Manila, which gives away all new rice varieties to those who can use them. There are also known cases where companies from industrialized countries gave away licenses to research institutions in LDCs without charging for them; this free transfer has involved genes, promotors and vectors.

Technology transfer and funding

Because technology transfer was a central issue of the meeting, many speakers addressed this question, from different angles: Cyrus Ndiritu (Kenyan Agricultural Research Institute), Robert Beachy and Claude Fauquet (International Laboratory for Tropical Agricultural Biotechnology, Scripps Research Institute, USA), Joel Cohen (International Service for National Agricultural Research, The Hague, The Netherlands) and, last but not least, the host of the meeting, Catherine Yves (Michigan State University, USA). For technology transfer to be successful, there need to be research and development programs in LDCs, as well as the possibility of training young and motivated researchers from these countries in competent cooperating host laboratories, usually located in the North.

Most of the direct funding of agricultural biotechnology in the Third World comes from the public sector, usually from development agencies such as the US Agency for International Development and the Consultative Group on International Agricultural Research or from the ministries of individual LDCs and industrialized countries. The Consultative Group on International Agricultural Research is itself subsidised by the World Bank and many donor countries, with some funding coming from independent foundations. Gary Toenniessen (Rockefeller Foundation, USA) reported that the Rockefeller Foundation has spent US$86 million on the rice program since 1988, of which a large part went to IRRI in Manila. Another source of funding was described by Nicolas Matéo (National Biodiversity Institute, Costa Rica), which has made contracts with various companies to help them get access to indigenous plants, animals or microorganisms. The best known partner in this arrangement so far is the pharmaceutical giant Merck. In all, the contracts have brought Costa Rica approximately US$ 2.5 million since 1991.

Introducing the products of a new technology in the poorest areas of the world is not an easy task and many obstacles still need to be overcome. However, not to develop and distribute the best possible seeds for crops on which millions depend for their livelihood would be a grave mistake. A bag of seeds can make a world of difference.

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New pharmaceuticals from marine organisms

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Definitions of 'marine biotechnology' often refer to the vast potential of the oceans to lead to new cures for human and animal disease; the exploitation of natural drugs has always been the most basic form of biotechnology. Although only initiated in the late 1970s, natural drug discovery from the world's oceans has been accelerated by the chemical uniqueness of marine organisms and by the need to develop drugs for contemporary, difficult to cure, diseases. Current research activities, while primarily within the academic laboratories, have generated convincing evidence that marine drug discovery has an exceedingly bright future.

There has always been a pressing need for the development of new pharmaceuticals. Even today, our inability to cure cancer, AIDS, Alzheimer's Disease and arthritis, to mention but a few, has demonstrated the continuing importance of new drug discovery. The growing incidence of drug-resistant infectious disease alone suggests that a major investment is needed now if we are to combat this growing problem. Of all the natural sources for drugs, the marine environment is clearly the last great frontier. Avoided, perhaps because of the difficulty of remaining underwater for sufficient time, marine ecosystems were only recognised recently as having the potential to contain a wealth of novel new drugs. Since the 1970s, chemists have been unravelling the fine structures of novel organic compounds produced by marine plants and animals. They were surprised to find a dramatically different environment for biosynthesis, one including new building blocks and incorporating unprecedented enzymatic reactions. The result was the recognition that marine sources possess significant potential to generate new drugs. Within the past few years, several collaborations have been constructed to encourage the interaction of academic marine science with drugs industries worldwide. While this interaction remains incomplete, the pharmaceutical industry now accepts the world's oceans as a major frontier for medical research. The emergence of this new field, sometimes called marine pharmacology, has been of enormous interest in the popular press and several review publications have appeared on the topic[1–5].

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Marine drugs and new insights into human disease

Since the early investigations of the 1970s, significant advances have been made in marine drug discovery. Academic researchers began to collaborate with pharmacologists and, in the 1980s, the potential of the oceans became clear, with many unique bioactive substances being extracted from marine plants and invertebrate animals[6–16]. Most of the compounds initially discovered were not effective in treating diseases, but some were found to possess important biochemical properties that have influenced our understanding of human diseases. These compounds, referred to as pharmacological probes, have the potential to revolutionise our understanding of the underlying biochemistry of disease. During this early period, unique marine molecules such as manoalide and okadaic acid were discovered. Manoalide, isolated in Hawaii and California from the sponge *Luffariella variabilis*[17,18] was the first substance ever observed to inhibit phospholipase A_2 selectively, an enzyme centrally involved in many inflammatory diseases. Okadaic acid, a cyclic polyether first isolated from the sponge *Halichondria okadai*, is a highly selective inhibitor of the enzyme protein phosphatase, allowing it to be used to probe basic cellular phosphorylation processes. Okadaic acid and manoalide are now commercially available for biochemical research.

Literally hundreds of bioactive metabolites have subsequently been discovered. Major programs now exist in the USA and Japan, and there are significant research programs worldwide. However, because of competition and secrecy within the drug industry, it is usually difficult to learn of the existence of promising new drugs. Given this, it is also difficult to determine just how many marine drugs are currently in development

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PII: S0167-7799(97)01081-0

for the broad range of potential therapeutic applications. It is almost certain that many marine-organism-derived agents are in various stages of preclinical development. For cancer treatment, however, the information is readily available from the US National Cancer Institute (NCI), which has a major commitment to the development of new anticancer drugs. Over half of the NCI's natural products programs involve exploration of marine sources, and their efforts are being richly rewarded. At least six marine drugs are currently scheduled for clinical evaluation under the auspices of the NCI, for example, ecteinascidin 743, a novel alkaloid isolated from the mangrove ascidian *Ecteinascidia turbinata*[19,20], bryostatin-1, a macrolide antitumor agent isolated from the common fouling bryozoan *Bugula neritina*[21], dolastatin-10, a linear peptide found in the sea hare *Dolabella auricularia*[22] and Halichondrin B, a complex anticancer agent first isolated from the sponge *Halichondria okadai*[23]. Other marine drug candidates are also entering the screening system and being evaluated in preclinical studies. Although clinical trials have not yet been set up, discodermolide, from the deep-water sponge *Discodermia dissoluta*[24], and curacin A, a compound isolated from the marine cyanobacterium *Lyngbya majuscula*[25], have promising effects on cancer-cell division. Curacin A and discodermolide interact with the spindle protein tubulin in a similar fashion to Taxol®, which is a clinically used drug. Because Taxol® has been found to be superior to older drugs in the treatment of certain forms of cancer, these new agents are thought to hold similar promise.

It is quite clear that marine compounds have the potential to treat a wide array of diseases in addition to cancer. The pseudopterosins, a series of diterpenoid glycosides isolated from the Caribbean sea whip *Pseudopterogorgia elisabethae,* show impressive anti-inflammatory properties on the skin[26]. The pseudopterosins modify the inflammatory response of human white blood cells and, in doing so, they control a major degradative component of the inflammation reaction. This property, shown to be effective with the natural extract of *P. elisabethae,* has led to the development of a new additive, most recently used by Estee Lauder in their 'Resilience' line of skin-care products. A derivative of the natural product, methopterosin, is currently under development for a wide variety of inflammatory diseases including arthritis, psoriasis and asthma.

What is the future for marine drug development?

The success of marine drug discovery has relied upon the enormous diversity of plants and animals found mainly in shallow waters throughout the tropical oceans. Here, species diversity is high and drug discovery can capitalise on the vast number of species represented. The collection of macroscopic marine organisms and their drug evaluation continues today with good success. However, there is no question that these resources are limited and that collectable marine organisms are likely to be almost completely explored within the next 20 years. Where will scientists turn to ensure a continuing flow of new drugs? The answer to this question is complex, as drugs can now be developed by many methods including computer-aided drug design and combinatorial synthesis. Drug discovery from nature, however, may survive, because of the massive numbers of microorganisms found in the oceans. By processes similar to the discovery of penicillin and the large numbers of antibiotics produced by soil microorganisms, the complex microorganisms found in the sea provide an immense opportunity to discover new drugs. Although almost completely undeveloped, there is now growing evidence that the complexities of marine-microbial growth and cultivation can be solved. Programs are now emerging in the USA, Canada and Japan to exploit marine microorganisms, and the results to date are highly promising[27]. Microorganisms are being isolated that are exclusively marine; they require seawater for growth and so appear to be restricted to that environment[28]. The complex microbial adaptations to growing in the ocean are fundamentally different from those in land-based organisms. Nutrients are scarce in the sea, forcing many microorganisms to associate with the nutrient-rich plants and animals found there. Microbial symbiosis is common and associational specificity high, and competition for resources at the microscopic level is intense. This has resulted in microorganisms that produce a variety of chemical compounds for defense and competition. These compounds form the foundation for the development of marine microorganisms as a major drug resource. Due to the differing environments in which they occur, many marine microbial compounds are chemically unique; for example, the anti-inflammatory salinamides[29] and the bromine-containing antibiotic marinone[30] exemplify the structural novelty observed in marine bacteria. Marine fungi, in particular, seem to be a major focus of several research programs in the USA and Japan. Many marine fungi are unique to the ocean and thus provide opportunities for the isolation of unique drugs. Although only a small number of potential drugs have yet been isolated from the cultivation of marine fungi, this is an area of particular promise.

How can the barriers between academic and industrial research be overcome?

The drug-discovery activities within the pharmaceutical industry still focus intensely on the terrestrial environment. This is not surprising, given that lines of communication between medical and marine researchers have never been firmly established. Marine environments are unfamiliar to most pharmaceutical companies, and so they have been hesitant to initiate independent programs. This is slowly changing as exciting molecules are isolated from marine organisms. The emergence of marine microorganisms as a huge resource is likely to attract even more attention. With an investment in research, it is clear that saline microorganisms can be readily cultivated and explored using simple modifications of the growth conditions

and culture facilities that have been developed for soil microorganisms over the past 60 years. Minor modifications of existing microorganism-based programs will provide access to a major resource, and so it seems reasonable to predict significant industrial interest in this area of marine drug discovery.

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The science and intricacy of environmental safety evaluations

Othmar Käppeli and Lillian Auberson

Safety issues in biotechnology are subject to considerable controversy in public debate. Public concern is focused in particular on the degree of uncertainty associated with inadvertent and unforeseeable impacts of the deliberate or accidental release of genetically engineered organisms into the environment. The objective of this article is to demonstrate that there is a factual, scientific basis to environmental safety issues, by means of which the real, case-specific hazards requiring serious consideration by scientists, representatives of regulatory agencies and the public can be distinguished from conjectural hazards and naturally occurring background processes.

The principles of biotechnology have been unknowingly followed for centuries, during the selection of higher-yielding plants, microorganisms and animals by breeding for particular phenotypic characteristics. For these technological activities, perceived as traditional by the general public, safety has never been a major matter for concern. In the past, the methods used to obtain the desired phenotype have relied on the same biological mechanisms now exploited with the tools of modern genetic engineering, enabling new variants of microorganisms and plants to be produced at a more rapid pace. The difference lies in the fact that genotypes and phenotypes can potentially extend outside the range of traditional genetics and conventional plant breeding. The techniques of modern molecular biology also provide greater potential for the production of important medical and agricultural products. For example, in the pharmaceutical industry, the production of proteins such as monoclonal antibodies and interferon makes use of genetically engineered mammalian cell lines, while the production of antibiotics and antiviral vaccines relies heavily on recombinant DNA technology for the selection of appropriate microbial strains. Outside the laboratory and contained industrial production units, genetically engineered organisms are being released into the environment for pollution control and agricultural applications.

Public unease towards environmental biotechnology applications is due, in part, to the ever-increasing number of modified organisms whose long-term fate in the environment cannot be determined on an absolute basis. Preceding the environmental release of any genetically modified organism (GMO), extensive safety evaluations are carried out in order to gain the approval of the relevant regulatory bodies and of the general public. The purpose of the existing safety guidelines is to assure that an organism is environmentally safe, according to the currently available expertise. However, safety (see Glossary) cannot be expressed in absolute terms; it is a relative concept more adequately defined in terms of tolerability and acceptability limits, and yet a rational, analytical approach towards environmental safety is possible and could provide the necessary foundation for constructive discussions on the real issues of interest to scientists, representatives of the regulatory community and members of the general public. A clear safety-assessment methodology, with established scientific consensus on assumptions, data and methods, would also help to inspire public trust and confidence in biotechnology safety management[1].

The analytical rationale of safety assessments can also be useful in evaluating the impacts associated with the release of naturally occurring microorganisms. Most emerging infections are caused by pathogens that are normally present in the environment; these pathogens may become serious health threats when the ecological or environmental conditions encourage pathogen proliferation and spread. As a result, the incidence of emerging infectious diseases is likely to increase suddenly, possibly extending to other geographical areas. Safety assessments may be invaluable in uncovering the underlying factors of epidemic diseases, such as population shifts, changing human behavior, changing agricultural and food practices or changing ecology and climate[2,3]. In particular, the technique of scenario

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PII: S0167-7799(97)01083-4

elaboration could be used to establish the plausible event sequence leading to disease, from the initiating event to the designated endpoint. The definition of appropriate preventive measures for eradicating a health risk is dependent on the understanding of these causal chains. Human activities have, throughout history, been a major cause of modifications in the environment, which invariably affect the growth and abatement cycles of organisms that are naturally present.

Background

The regulation of human activities is based on the avoidance of risks, which is why environmental risk considerations have been an important consideration when dealing with GMOs. Environmental risk assessment and management procedures have relied heavily on methodologies developed for toxic chemicals in the environment. While there is reason to accept that the rationale of toxic-compound risk assessments is applicable to the evaluation of contained, hazardous industrial processes with GMOs, there is also reason to suggest that the planned environmental release of GMOs, for example, in plant-pest control or pollution abatement, requires another approach. In closed applications, the hazard potential is readily identifiable and arises mainly from the pathogenicity of the strains used, analogous to the toxicity hazard of chemical compounds[4]. However, when genetically engineered crop plants or modified microorganisms are released into the environment, it is not immediately obvious what the real hazards are.

In a formal sense, risk assessment can be defined as the use of scientific methods, models and data to obtain information for the four-stage process of hazard assessment, dose-response assessment, exposure assessment and risk characterisation[5]. A risk assessment of a technological application calculates the conditional probabilities of human harm, or risk, occurring from known hazards (see Glossary)[6]. During the risk-management process, appropriate control schemes aimed at reducing the risks to acceptable levels are implemented, along with cost–benefit analyses for determining the overall feasibility. The risk-assessment–risk-management methodology is an accepted approach for analyzing and predicting the potential damage to humans and the environment from technological applications requiring the use of toxic compounds, because it is possible to model and obtain quantitative data for the behavior of chemical substances in different environments and organisms. However, a risk-based approach is not necessarily the most appropriate for evaluating the environmental safety of GMOs. First, structure–activity relationships do not exist for organisms; the possible phenotypes expressed by a finite genetic code are endless. For example, in genetically engineered and conventionally bred crop plants, the expression (phenotype) of genetic information (genotype) in unexpected ways may result in potential harm to humans (through toxicity or allergenicity of the edible plant product) or to delicate ecological systems. Second, experience has not yet resulted in unconditional agreement about the real hazards of GMO use in the environment. For example, the question of whether the proliferation of GMOs or the spread of unwanted (inserted) genetic information to wild-type organisms are realistic hazards requires careful consideration before a risk analysis can be carried out.

Glossary	
Impact aspect	is a probable future outcome that inspires unease or apprehension
Danger or **hazard**	means a state, condition or process with an undesirable or injurious outcome; a source for **risk**[23].
Damage	refers to the impairment of value or function due to the occurrence of a **hazard**.
Risk	is a quantitative measure of the conditional probability of harm; it is a function of both the probability and the extent of **damage**[23].
Safety	is a relative term and is defined by tolerability and acceptability levels.
A theoretical definition:	A state, condition or process with complete absence of any **hazard**; not achievable in real-life situations.
A working definition:	A state, condition or process recognised as associated with tolerable **hazards** or acceptable **risk** not significantly different from accepted background levels[24].

Most of the GMOs intended for release are modified strains of known plants or microorganisms, either naturally present or with established records of safe use in the environment. An environmental safety evaluation of a GMO application should therefore include in its methodology a framework for 'discovering' potential hazards originating from the insertion and expression of foreign genetic information in wild-type parent strains. This framework would define the target and nontarget impacts relevant to the environmental use of a GMO, the potential hazards from these impact aspects and whether the identified hazards are intrinsic to the technology or are comparable to normally accepted (safe) outcomes of background life processes. The recognition that the safety of GMOs needs to be addressed before any risk analysis can take place suggests that environmental safety evaluations should first be safety-oriented before any risk analysis can take place[1].

The environmental-safety-evaluation methodology discussed in this article is therefore based on a two-stage procedure of safety assessment and risk analysis (Fig. 1). In this methodology, the objective and scientifically based safety assessment is dissociated from the value-oriented decisions of the risk analysis. The purpose of the safety assessment is effectively to manage and communicate the scientific information needed to evaluate the tolerability of potential damage from certain hazards that may occur from GMO use in the environment. This is accomplished in three interdependent stages during the safety assessment –

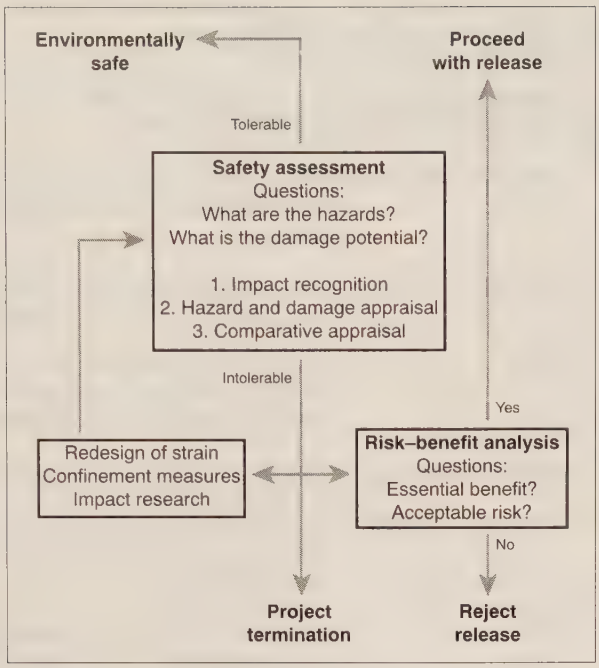

Figure 1
Environmental-safety-assurance methodology for the release of genetically modified organisms.

(1) impact recognition, (2) plausible hazard and damage scenario elaboration, leading to (3) an explicit statement on safety based on comparative appraisal strategies. A GMO can be declared environmentally safe exclusively from the safety assessment; by the same token, the GMO may be declared unacceptably hazardous and its intended application abandoned. In reality, the conclusions of the safety assessment are more nuanced. In some cases, the safety profile of the GMO can be improved by further development, or a subsequent risk analysis may provide the final word on the implementation of a particular application involving a GMO. In practice, risk analyses are rarely implemented in environmental safety evaluations; an organism labeled with a high potential hazard from the safety assessment is not considered for a risk analysis unless the benefits from its use can be demonstrated to be significant.

Safety assessment

Impact recognition

An impact aspect related to environmental safety is a potentially hazardous outcome of the release of GMOs (Box 1). The goal of impact recognition is to translate vague fears about the results of the release of a GMO into distinct concepts that will provide the basis for scientific and value-based judgements on desirability or undesirability, within the framework of comparable background processes. There are no absolute rules governing the negative and positive aspects of a GMO release; the impact aspects cover immediate and long-term environmental effects. For genetically engineered plants, impact aspects could include: increased allergenicity of crop plants receiving genes from another plant species; the loss of genetic diversity through the widespread use of modified crops; the potential poisoning of native nontarget animals, fungi or bacteria by toxins expressed by engineered pest-resistant crops; and the unwanted vertical or horizontal transfer of genetic information (e.g. antibiotic and herbicide resistance) from crop plants to weeds or bacteria. Impact aspects deal with hypothetical ecological roles and effects of an organism. In the 1980s, plans to spray engineered *ice⁻ Pseudomonas syringae* on crop plants, in order to prevent ice nucleation and subsequent frost damage[7], were initially thwarted by protesters, who were concerned about the possible climatic impact of the widespread use of the engineered bacteria[8].

Unlike classical hazard and exposure assessments for chemical processes, which make extensive use of the available structure–activity relationships for predicting toxicological and chemical behavior in different environments, biotechnological impact-recognition methods cannot rely on analogous genotype–phenotype relationships to forecast the outcome[9]. The unpredictability of the phenotype of an organism from its genotype alone, especially its ecological traits and population dynamics, is a major concern for environmental releases of GMOs[10]. Once released, organisms become involved in the natural cycles of proliferation, decline and evolution, driven by environmental factors such as microclimate and nutrient availability.

There can be a lack of consensus among specialists as to which impact aspects are relevant to safety assessments. For example, GMO persistence and spread are contextual impact aspects. In applications such as the bioremediation of contaminated sludge or soil, achieving the target effect (pollution abatement) would be expected to be proportional to the population of viable cells available. Therefore, persistence and spread of the pollution-controlling species would be desirable. In contrast, the persistence of conventional or genetically modified crop plants may be undesirable, as they would produce plants in succeeding crops, decreasing the homogeneity of the main seed. There is no doubt that more knowledge about the fundamental principles of ecology is needed. In any case, impact assessment must be adapted on a case-by-case basis[11]. As a guideline, it is recommended that all environmental impact aspects, especially those characterised by the greatest variability and uncertainty, be enumerated as objectively as possible[12]. The ultimate endpoints for defining the scope of impact recognition, however, are the published regulatory guidelines that must be fulfilled prior to obtaining regulatory approval for environmental releases (Box 1)[13].

Hazard and damage scenarios

Once the most important impact aspects of an environmental release have been identified, their influence on hazardous and actually harmful (see Glossary) future outcomes needs to be explored. The 'scenario' method is well suited to evaluating long-term impacts and is gaining advocates for its systematic use in technology assessment[14,15]. A scenario is defined as the description of all possible causally or conditionally

Box 1. Regulatory information requirements for the environmental release of genetically modified organisms, adapted from European Union directive 90/220/EEC[13].

Characteristics of the modified organism
(a) Methods used for the modification.
(b) Methods used to construct and introduce the insert(s) into the recipient or to delete a sequence.
(c) Description of the insert and/or vector construction.
(d) Purity of the insert from any unknown sequence and information on the degree to which the inserted sequence is limited to the dna required to perform the intended function.
(e) Sequence, functional identity and location of the altered, inserted or deleted nucleic acid segment(s), with particular reference to any known harmful sequences.

Potential environmental impacts
(a) Potential for excessive population increase in the environment.
(b) Competitive advantage of the genetically modified organism over the unmodified recipient organism.
(c) Identification and description of the target organisms.
(d) Anticipated mechanisms and results of interaction between the released genetically modified organism and the target organisms.
(e) Identification and description of nontarget organisms that may be affected by accident.
(f) Probability of shifts in biological interactions or host range after release.
(g) Known or predicted effects on nontarget organisms in the environment, impact on population levels of competitors – preys, hosts, symbionts, predators, parasites and pathogens.
(h) Known or predicted involvement in biogeochemical processes.
(i) Other potentially significant interactions with the environment.

related states, events and actions, whether they extend into the future or the past. Scenario elaboration is a complex undertaking that resists generalisation into algorithm form. Because scenarios deal with hypothetical states, the reliability of a scenario should be judged on its consistency and plausibility, which can only be determined through good, intuitive scientific judgement.

Hazard and damage scenarios can be considered to be compact summaries of the safety-assessment process, in which the sequences of causal events and relationships leading to harmful outcomes from specific hazards are exposed in a diagram. Starting from the working hypothesis that the release of a GMO into the environment is potentially hazardous, two separate classes of events can be described: inductive reasoning is used to imagine all probable, independent causal event chains initiated by the hazard that might lead to damage in the future (Fig. 2); deductive reasoning is used to establish, retrospectively, all the possible independent pathways leading to the same (hazardous) outcome from known processes. All events in a scenario possess unique probabilities of occurrence; thus the probability of a particular causal pathway occurring is the product of the probabilities of each independent event. While both the retrospective and the prospective branches of the pathways are important, they serve different purposes – the inductive approach is used to assess the damage potential associated with a specific hazard, and the deductive approach is used to derive safety conclusions about this hazard, based on comparisons with the threshold hazard potential from known background processes.

Experimental information can provide the basis for the analytical logic needed to construct the sequence of events arising from a hazard that lead to damage. Sources of information include: studies on the ecological impacts of potential target and nontarget effects of an organism within a laboratory model ecosystem; mathematical models; field tests; and fundamental research (e.g. physiology, metabolism, genetics) with the GMO.

An example of a scenario for damage potential is given in Fig. 2 for the safety assessment of genetically modified cotton plants expressing a *Bacillus thuringiensis* subspecies *kurstaki* protein toxin specific for lepidopteran insect pests and a marker gene for herbicide resistance (the scenario is similar for antibiotic-resistance marker genes). If one of the impact aspects is assumed to be horizontal gene transfer, then a potential hazard could be the unwanted spread of genes into the environment by transformed soil microorganisms. It should be noted that, although the actual probability of horizontal gene transfer from plants to microorganisms is extremely low to nonexistent, it is assumed to occur (probability = 1) for the purposes of estimating the damage potential. One possible event cascade can be imagined: rotting plants liberate transgenic DNA into the soil[17]; through the process of transformation, microorganisms present in the soil pick up the genetic information for *B. thuringiensis* toxin and herbicide resistance; recombination events then allow microorganisms to integrate the DNA into their genome (for a summary of the literature on DNA persistence and horizontal transfer from transgenic plants see Ref. 18). The resulting damage, which must be assessed for tolerability, is the ecological consequences of the inadvertent spread and persistence of unwanted traits within microorganism populations. A similar event cascade can also be constructed for the spread of unwanted traits from soil microorganisms to other plants through root-cell transformation. Once the genetic information has successfully crossed the species barrier, there is the potential for the spread of the unwanted traits to other plant species. Because

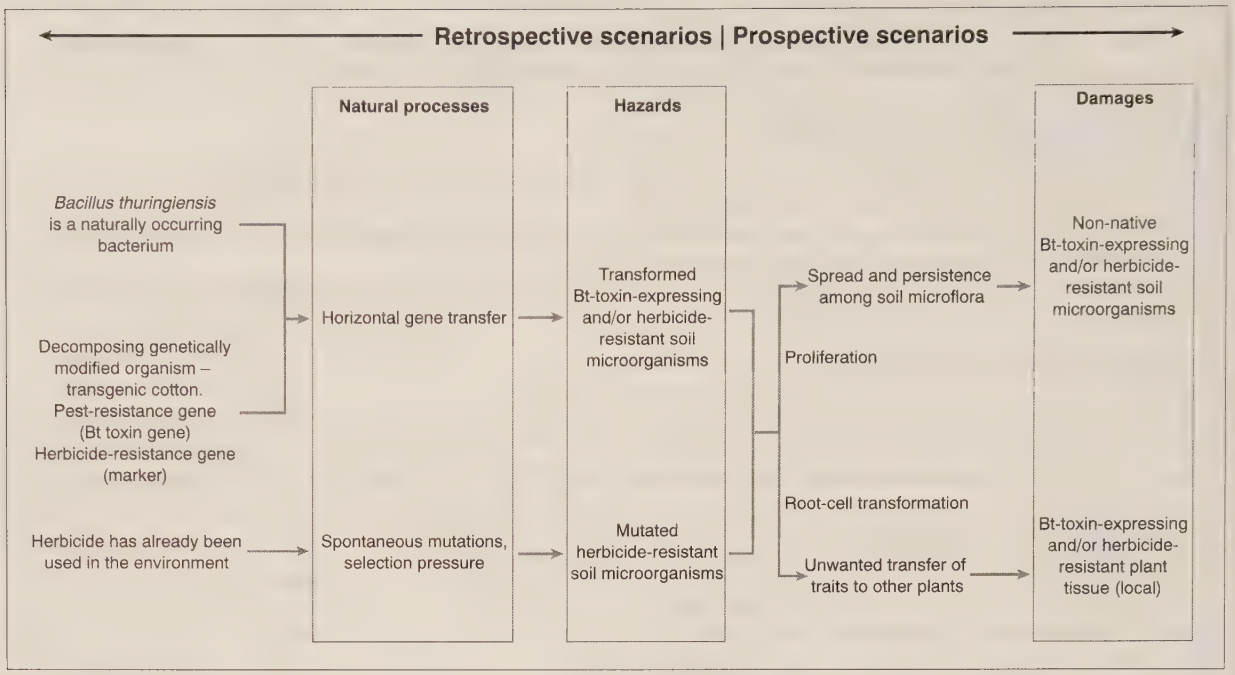

Figure 2

An example of a hazard-and-damage-scenario diagram for gene transfer from transgenic cotton plants to soil microorganisms or to other plants. The potential hazard (see Glossary) is the emergence of recombinant soil microorganisms. On the right hand side of the diagram are the possible causal event chains that could lead to damage if the hazard were to occur; on the left hand side are the independent scenarios that lead to the same potential hazard. Although the actual probabilities of the individual events of each causal chain are extremely low to nonexistent, the pathways are assumed to occur for the purposes of estimating the damage potential. Examples of genetic traits adapted from Ref. 16.

weeds are potential candidates for receiving transgenic DNA, the damage arising from the transfer of herbicide resistance to weeds needs to be evaluated.

Perceptions of hazard and safety change with technological knowledge. In the early days of safety assessment, the mechanism of horizontal gene transfer would have been considered a hazard in itself rather than an impact aspect. It is now accepted that horizontal gene transfer is a ubiquitous mechanism in nature, thereby shifting the focus of safety assessments to the real issue of concern: the damage due to the potential spread of unwanted genetic traits through this naturally occurring mechanism.

Comparative safety appraisal

The final step of the scientific safety assessment appraises the safety of a GMO based on observations and analogies with similar organisms, systems and processes for which quantitative data or experience are available. At the heart of this procedure is the familiarity principle, which was the first of three criteria in the GMO assessment framework endorsed in 1989 by the US National Academy of Sciences[4,19]. Evaluation of familiarity is performed by asking the following questions[20]: how familiar are we with biotechnological applications of this type? with the starting materials? with the behavior of similar organisms in the environments for which the GMO is intended? If the GMO, its target use and its environment of release are considered 'familiar', then sufficient knowledge would be available for a qualitative safety judgement. According to Persley *et al.*[20], '[t]he familiarity criterion is central to the suggested evaluation framework. It permits decision makers to draw on past experience introducing plants and microorganisms into the environment, and it provides flexibility.' They caution, however, against misinterpreting 'familiar' as 'safe'.

A GMO considered familiar usually implies that it was derived from a conventional wild-type parent strain of known phenotype, for which there is a history of safe use in the environment. The comparison of the modified and parent strains should highlight potential secondary, unintended genomic modifications that may be present in the GMO, such as pleiotropic effects, phenotypic variability and changes in cellular metabolism. These modifications are also manifest in conventional strains at background levels, for all organisms naturally evolve and respond to selection pressure through genomic changes, which are the cause of the modifications described. The awareness and evaluation of unintended modifications in a GMO are important for the safety assessment; however, it is the magnitude of these effects, compared with accepted background levels found in unmodified counterparts, that determines safety. Because safety cannot be expressed in absolute terms, a GMO is judged 'as safe as' a familiar organism, whose environmental safety record can then be used to make reasonable inferences about the environmental safety of the GMO.

In the example of horizontal gene transfer from transgenic cotton plants in Fig. 2, application of the familiarity principle might indicate that the two

background pathways, depicted as retrospective scenarios having low actual probabilities of occurring but yielding a similar hazard potential to the spread of pest- and herbicide-resistance genes from the GMO. For damage-estimation purposes, these alternative pathways are assumed to occur, suggesting that the genetic information introduced into the cotton (*B. thuringiensis* toxin, herbicide resistance) has always existed in the environment. The transformed and mutated soil microorganisms that may harbor the unwanted *B. thuringiensis*-toxin and herbicide-resistance traits obtain these genes by gene transfer from naturally-occurring *B. thuringiensis* or spontaneous mutations under selective pressure introduced by the use of herbicide in the environment. Therefore, the hazard and damage from the emergence of transformed, mutated soil microorganisms retaining unwanted traits could result from pathways other than GMO release; the trait potentially transferable from the transgenic cotton plant to the environment do not represent novel genetic information for the surroundings, nor a measurable aggravation of an existing, tolerated background state.

Even in the absence of the deliberate releases of GMOs, the environment is the stage for ecologically diverse populations of natural organisms subject to the ubiquitous mechanisms of mutation, gene transfer and recombination. The determination of threshold hazard levels, based on background states and mechanisms, is dependent on the degree of sophistication of existing scientific techniques; it is not always possible to obtain information solely through experimentation, especially in relation to long-term environmental effects. Tolerability and acceptability criteria cannot be dissociated from society's value system, and so the environmental safety assessment may be publicly debated, but this should not be allowed to stray too far from the scientific evaluation.

The iterative loop

If a GMO does not meet the familiarity criterion, this does not necessarily mean that its application should be suspended. Failure to establish familiarity implies that the empirical information base acquired through experience with other organisms, processes and systems is insufficient for providing reliable predictions about the safety of a GMO. The role of the iterative loop in the scientific safety assessment is to accommodate the case-by-case nature of GMO environmental safety considerations and to render the entire methodology more comprehensive and interactive. The goal of each iterative cycle is to minimise the damage potential arising from plausible hazards. Specific actions may be carried out for concrete incremental safety gains, such as strain redesign or confinement within predefined geographical limits, in combination with dependable monitoring techniques (Fig. 1). Further impact research may also be necessary, although progress here may require parallel development of adequate technological tools for investigating and mitigating the possible nontarget behavior of a GMO in its environment of release.

The activities of the iterative loop feed back to the safety assessment, which is performed anew with acquired knowledge or with defined contingency plans. When the safety profile of a GMO can no longer be improved and the safety assessment still cannot guarantee the environmental safety of a GMO, then the project might be abandoned for lack of safety or evaluated for overall benefit versus risk.

Risk analysis

Traditionally, risk is calculated as the product of the extent and probability of damage. For biotechnological applications, the potential damage from the environmental release of a GMO is weighed against the potential for benefit in a risk analysis. While the iterative stage of the safety assessment and the management stage of the risk analysis both share the common objective of decreasing the overall level of risk, there are inherent differences as to how this is achieved. Risk is minimised in the safety assessment by decreasing the damage extent in the risk equation (iteration), whereas the probability of the damage occurring is reduced to low levels through effective risk-management or control techniques.

The hazard and damage scenarios from the safety assessment define the parameters and assumptions that need to be studied in more detail during the risk analysis. A list of the experimental information that would be needed for determining the likelihood that, for example, unwanted traits from transgenic plants are spread to their environment through horizontal gene transfer would include: duration of plant-DNA persistence in soil; mechanisms of gene transfer from plant to soil microorganisms and vice versa; and the inherent rates associated with plant and microbial genetics, physiology, reproduction and metabolism. In experiments conducted to determine the probability of horizontal gene transfer from plants to microorganisms, it was demonstrated that this mechanism is associated with a low likelihood of occurrence, even when microorganisms were subjected to selective pressure[21].

Until now, most decisions concerning the environmental safety of GMOs have been made on the basis of the safety assessment alone. In most cases, the identification of any realistic hazard associated with a biotechnological application was sufficient grounds for stopping a project in its early stages, avoiding any risk. This is illustrated by the insertion of a gene encoding a sulfur-rich protein from the Brazil nut (*Bertholletia excelsa*) into methionine-deficient legume crops for use as livestock feed. Methionine-poor soybean meal used as animal feed must be supplemented with this amino acid to ensure proper animal nutrition. As seeds of the Brazil nut are unusually high in sulfur-containing amino acids, the isolation and transfer of a gene encoding a sulfur-rich protein into soy beans would improve the nutritional quality of this seed protein. In one study on the potential allergenicity of transgenic soy beans, it was found that a 2S albumin expressed by the transgene binds human IgE from the sera of individuals allergic to Brazil nuts[22]. It was therefore suggested that

the 2S albumin was the major allergen from the Brazil nut. In the light of such studies, it was concluded that the risk of allergic reactions in humans, who could have potentially consumed the modified soy beans, was greater than the nutritional and economic benefits of an amino-acid-balanced animal feed. The project was abandoned.

The analysis of risk is central to any technological debate, although the definition of risk (delayed action, catastrophic, severe, tolerable, unknown) and the perception of risk may vary according to an individual's awareness, values and motivations. For environmental biotechnological applications, reliable risk analyses would require more scientific knowledge and experience than current experience can provide. Moreover, the ordinary risk equation is not satisfactory for potential hazards associated with the release of GMOs in the environment. Even though there is a low probability of a hazard occurring, its frequency of occurrence may still be observable with increasing use of a GMO in the environment.

Conclusions

History has shown that biotechnological processes are closely entwined with human subsistence. In this modern era of science, precise molecular-biological techniques are beginning to replace traditional techniques for the modification and selection of organisms with desirable phenotypic characteristics and, although the techniques are novel, the biological mechanisms exploited are not.

The enthusiasm and expectations of the public for applications from the biotechnology sector have been offset by wariness regarding the potential for adverse effects on humans and the environment. Environmental safety evaluations are positioned at the interface between technology and society, and could provide a much-needed constructive basis for focused discussions between scientists, representatives of the regulatory bodies and the general public. The classical safety and risk analyses applied to toxic chemicals and the older technologies are only partially applicable to assessing the long-term environmental impacts of the release of GMOs. Organisms do not have foreseeable genotype–phenotype relationships analogous to the structure–activity relationships for toxic chemicals, which allow certain quantitative predictions of toxicity hazards and risks to be made.

Environmental safety evaluations are difficult and must be accomplished within the limits of technological knowledge, while fulfilling the dual requirements of comprehensiveness and comprehensibility. One of the objectives of this article has been to demonstrate that the science-based safety assessment can be kept distinct from the value-based risk analysis in environmental safety evaluations. This separation of tasks is essential for constructive safety discussions based on scientific objectivity with accurate definition of realistic hazards. The safety-oriented environmental safety evaluation identifies the impact aspects of GMOs released into the environment, their potential hazards, and makes reasonable inferences about the safety of GMOs based on previous experience with familiar organisms having a history of safe use. It is possible to determine the environmental safety of a GMO solely from the safety assessment, and experience has shown that the decision framework for biotechnological application has been safety-based rather than risk-based. When necessary, risk analysis is performed in environmental safety evaluations, but the difficulties in obtaining quantitative predictions of risk are a reflection of the complex interactions of an organism with other organisms and its surroundings, and of the changing societal values on the benefits and risks of technological achievements.

The final objective of this article has been to illustrate the power of the hazard-and-damage-scenario method in safety assessment. Scenario design is gaining acceptance as a valid method for technological assessments; it has been recognised as an effective interdisciplinary tool for predicting likely outcomes by examining all the possible permutations of future outcomes. Although they are qualitative, scenarios succeed in integrating relevant quantitative data from a wide variety of scientific sources. Retrospective scenarios are useful for highlighting parallel background processes that result in similar outcomes. The scientific definition of threshold hazard levels, based on normative, parallel background processes, is necessary in keeping the safety discussion on a factual basis.

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Enzymes involved in the processing of starch to sugars

W. Douglas Crabb and Colin Mitchinson

The commercial processing of starch to mono- and oligosaccharides depends on the availability of three major enzymes – glucoamylase, alpha-amylase and glucose isomerase. Each of these enzymes has a unique pH and temperature optimum for use, and so unit operations of a starch plant reflect the varying operating conditions for each individual enzymatic step. This article will discuss how recent advances in molecular biology and protein engineering have allowed enzymes with improved operating parameters to be introduced to commercial applications, providing the starch processor with enhanced plant efficiency, lower operating costs and higher-quality products.

Starch from cultivated plants represents one of the most ubiquitous and accessible energy sources on the planet. Although corn (maize) is the major crop used by starch processors, other sources, such as wheat, rye and potato, are becoming more significant. The major use of processed starch is for the production of glucose, which is subsequently used to produce crystalline dextrose, dextrose syrups or high-fructose corn syrups (HFCS). Alternatively, the glucose may be fermented to produce other products such as ethanol, amino acids or organic acids. Because fructose production is the kernel of the corn wet-milling industry, this article will discuss those enzymes involved in the processing of corn to HFCS and the application of technology to improve processing options. The basic steps described below are common to all HFCS processes, and the reader should refer to Fig. 1 as each part of the process is discussed. More detailed treatment of the variety of industrial starch processes and the commercial enzymes (Fig. 2) used are available within two recent comprehensive books[1–4].

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Liquefaction

The object of starch liquefaction is to convert a concentrated suspension of purified granular starch into a solution of soluble, shorter-chain-length dextrins. The starch slurry is adjusted to approximately 30–35% dry solids and passed through a high-temperature heat exchanger (jet cooker) that instantaneously raises the temperature of the slurry to the gelatinization temperature (for corn starch, 70–90°C). As the anhydrous starch granules begin to swell irreversibly and the amylose in the granules becomes soluble and is released, the viscosity of the solution increases dramatically. To counteract this and provide a suitable substrate for subsequent saccharification, the starch must be partially hydrolysed. Over the past 30 years, almost all processors have replaced the original method (acid hydrolysis at 140°C or higher) with the addition of an alpha-amylase (AA) prior to jet cooking, thus improving the process and the product. AA (α-1,4-glucan-4-glucanohydrolase; E.C.3.2.1.1) hydrolyses internal α-1,4-glycosidic linkages in starch, essentially at random, to give shorter maltodextrins. In order to assure the removal of all lipid–amylose complexes, the preferred temperature of gelatinization is above 100°C. This has driven the replacement of the AA from

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PII: S0167-7799(97)01082-2

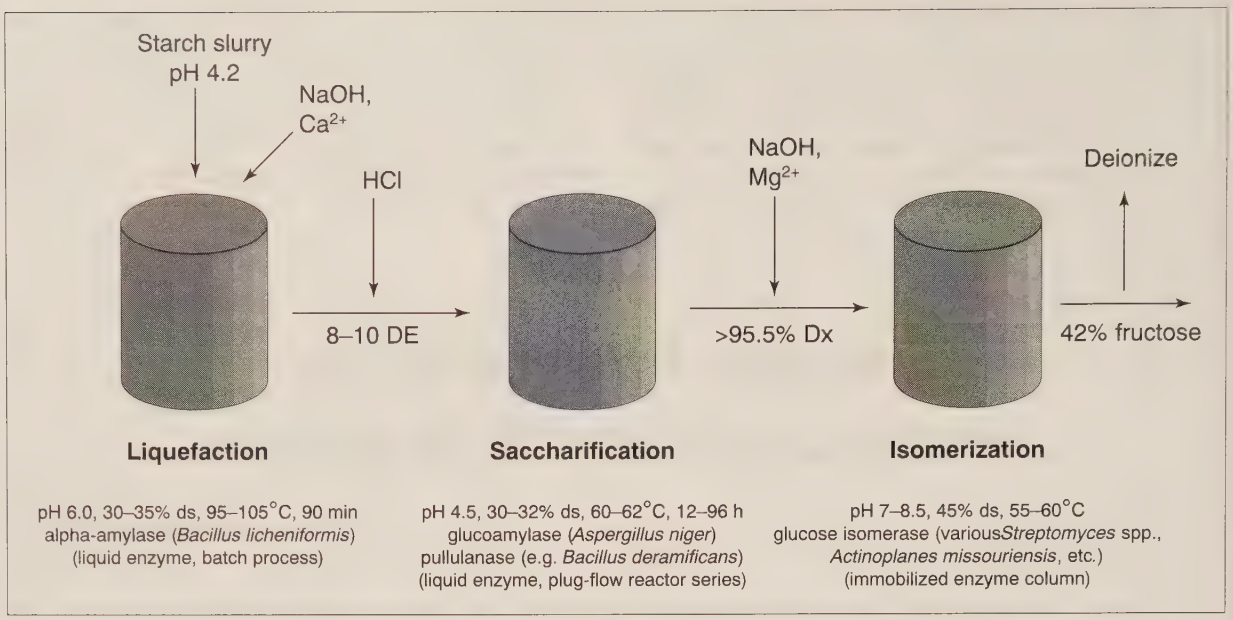

Figure 1

The starch process for high-fructose corn syrup. Schematic outline of the enzymatic steps in the processing of slurried corn starch to fructose, showing individual enzyme-usage conditions and typical processing parameters. Arrows indicate adjustment points within the process for pH and/or ion components. The process parameters may be different when producing ethanol from corn. The term 'ds' refers to the percentage of starch or glucose dry solids suspended in the slurry. DE is 'dextrose equivalent', a measure of the number of reducing ends present in a starch hydrolysate; each reducing end of an oligosaccharide is equivalent to a single dextrose residue. The greater the degree of starch liquefaction or hydrolysis, the higher the DE. Undegraded starch has a DE approaching zero; a fully hydrolysed starch would have a DE of 100. DE is related to average chain length of the oligosaccharide by the following formula: $DE = 180/(162n + 18) \times 100$, where n is the average oligosaccharide chain length. For example, a starch slurry with a DE of eight has an average chain length of ten glucose residues. The term '%Dx' is the percent of dextrose in the solution. In the example shown, after saccharification, the process stream would have 32% dry solids with greater than 95.5% dextrose (DX).

Bacillus amyloliquefaciens with the more thermostable enzymes from *Bacillus stearothermophilus* and *Bacillus licheniformis*. Although these enzymes are able to operate briefly at temperatures as high as 105°C, the process cannot be performed much below pH 5.9 as AA thermostability decreases at lower pHs. To be compatible with the pH optima of these preferred liquefaction enzymes, the pH of the starch slurry is adjusted from its natural pH of 4.5 to pH 5.8–6.5, and Ca^{2+} is added to improve enzyme stability. The next process step (saccharification) requires pH adjustment back down to pH 4.2–4.5. These adjustments increase the chemical costs and require additional ion-exchange refining of the final product for salt removal.

An AA able to operate at lower pH would reduce these costs, simplify the process and reduce high-pH byproduct (e.g. maltulose) formation in the liquefact. Amylases from extremophiles, especially thermoacidophiles, have promising properties[5–11], but none have been produced at commercially viable levels. One approach to liquefaction at lower pH, solving this problem, used a mixture of the enzymes from *B. stearothermophilus* and *B. licheniformis*[12]; another, protein engineering of the *B. licheniformis* AA, resulted in an enzyme able to operate at lower pH, lower calcium levels or higher temperatures[13]. Figure 3a shows the relative abilities of engineered and wild-type *B. licheniformis* AA to cause starch depolymerization at low pH. This same variant enzyme was also shown to be more effective than the native amylase at high temperature (107°C) across a range of Ca^{2+} concentrations (Fig. 3b) or over a range of temperatures (Fig. 3c). Thus, the engineered amylase is a generally more robust enzyme that increases the range of viable plant-operating parameters. Recently, the crystal structure of *B. licheniformis* AA has been published[14].

After jetting, the starch mixture is held at 95–100°C for up to three hours to allow the enzyme to hydrolyse the starch to an average chain length of 8–12 glucose units, thus producing the preferred substrate for saccharification.

Saccharification

The process of removing single glucose residues from a soluble oligosaccharide is termed saccharification and, in its simplest form, is catalysed by an exo-acting 1–4-glucanohydrolase (glucoamylase; E.C.3.2.1.3; GA), which sequentially removes a glucose unit from the nonreducing end until all of the oligosaccharide is degraded to glucose. The enzyme of choice is isolated from *Aspergillus niger* or a closely related species, for example *A. awamori*. *A. niger* GA has a pH optimum near 4.2, is extremely stable at 60°C, and is produced at high levels by industrial fermentation processes.

For saccharification, the pH of the liquefact is adjusted down to 4.2–4.5 and the dry solids are diluted to 30–32%. This pH adjustment serves two functions:

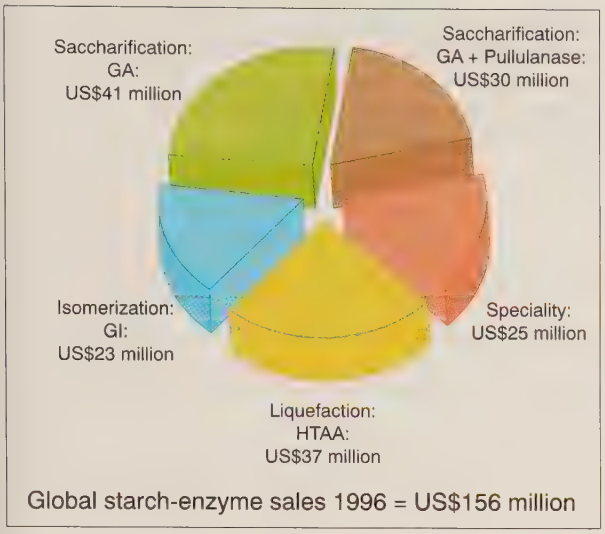

Figure 2
The global market for starch enzymes in US$. Enzymes used in the processing of starch and their relative value in the market. Speciality enzymes include acid fungal alpha-amylases, low-temperature bacterial alpha-amylases, beta-amylases and transferases. GA, glucoamylase; GI, glucose isomerase; HTAA, thermostable alpha-amylase. The GA market includes both straight GA for ethanol production and blends of GA with pullulanase for high-fructose corn syrup, and these are indicated separately.

first, it stops further action of the AA so that the liquefact maintains an average chain length optimal for saccharification; second, it moves the pH closer to the optimum for the GA. The saccharification reaction is most often performed in a series of plug-flow reactors, with the enzyme dosed so that peak dextrose is achieved between 24 and 96 hours at 60°C, depending upon plant capacity needs.

There are some practical difficulties in this process; for example, in natural starch sources, the substrate is a mixture of both amylose (1–4 linkages) and amylopectin (branched 1–6 links). Glucoamylase is efficient at cleaving the 1–4 links, but when it reaches a 1–6 branch point, the enzyme is slower to hydrolyse the bond and the result is a build-up of isomaltose. One solution to this problem has been the use of a blend of enzymes containing both GA and pullulanase (E.C.3.2.1.41), which has the ability to break the 1–6 linkages. The key to success was finding pullulanase enzymes with the same pH and temperature optima as the GA. In general, the use of the blended products increases the glucose yield from 94% to over 95.5%.

A second practical problem is that the process conditions require high dry-solids concentrations (32%) to be economical, which results in high concentrations of glucose (>95%). Under these product-enriched conditions, the GA will tend to form reversion products. In the case of 1–4 reversion products, such as maltose, an equilibrium will exist at approximately 1–2%, but 1–6 reversion products will tend to accumulate in the reaction at the expense of glucose. The solution is to balance the dosage of the enzyme, the temperature of the reaction and the reaction time so that, at later stages, thermal inactivation of the GA results in little active enzyme remaining, leading to less reversion.

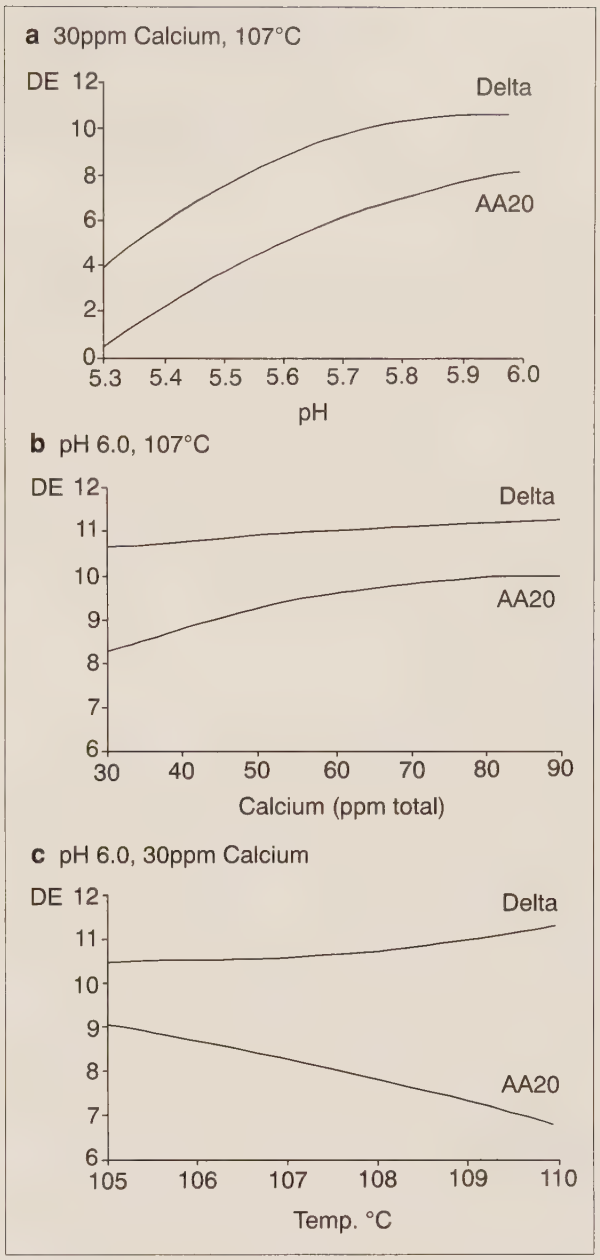

Figure 3
Improved starch liquefaction with an alpha-amylase variant. AA20 is Spezyme® AA20, a *Bacillus licheniformis* wild-type product. Delta is Spezyme® Delta AA, a *B. licheniformis* variant product. For liquefaction, a 35% dry solids wet-milled corn-starch slurry was adjusted to the indicated pH and Ca^{2+} levels. Enzyme samples were produced by fermentation of a *B. licheniformis* host containing either the native or variant amylase gene. Equal activities of the variant or native amylase were added to the slurry prior to jet cooking at the described temperatures. The dextrose equivalent (a measure of the number of reducing ends in the solution) was determined by measuring reducing-sugar content of the slurry following a 90 min hold at 95°C.

One future goal in saccharification is improved thermostability of GA[15], to allow reactors to be run at higher temperatures and dry-solids levels for shorter time periods. Engineering a GA that has a decreased ability to hydrolyse (and therefore revert) 1–6 linkages could

prove highly effective in combination with improved pullulanase products (P. Coutinho, PhD Thesis, Iowa State University, USA, 1996). The crystal structure of *A. niger* GA has been recently determined[16] and the catalytic and starch-binding domains identified[17,18].

Isomerization

Following saccharification, the dextrose mixture is refined, carbon filtered, concentrated to >40% dry solids and the pH adjusted to a more neutral level (pH 7–8). An enzymatic step isomerizes the sugar aldehyde (D-glucose) to the keto form (D-fructose) by passing a concentrated glucose syrup over an immobilized column that has active glucose isomerase (D-xylose-ketol isomerase; E.C.5.3.1.5; GI) attached to a solid support. (NB Although the preferred substrate of this enzyme is xylose, the name glucose isomerase is more commonly used.) The use of an immobilized enzyme allows a continuous process and avoids the introduction of the enzyme into the product. In some cases, the support is as simple as GI-producing cells crosslinked to each other with gluteraldehyde. In other cases, the immobilized column consists of GI that has been partially purified and attached to a cellulosic resin, or of crosslinked GI crystals. The flow rate across the column is adjusted to give a retention time that results in a product stream consisting of 42–45% fructose. Several enzymes are in commercial use and, regardless of the source of the enzyme, the methodology is similar. The typical reaction conditions are pH 7–8.5 and temperatures of 55–65°C, with a fructose solution of 55% being the preferred commercial product. The thermodynamics of the operating conditions only allow a fructose solution of approximately 42% to be reached; in order to produce the 55% HFCS, a portion of the fructose stream is fractionated to yield 90% fructose syrup, which is then blended back to make a 55% fructose final product. A detailed review of the industrial GI process and enzyme technology has recently been published[19].

Direct conversion of the glucose syrup to 55% fructose would simplify the process. Theoretical calculations show that isomerizing at elevated temperatures drives the equilibrium towards fructose, and experimental data support this. However, to convert glucose directly to fructose at a final fructose concentration of 55%, the reaction temperature must approach 110°C (Ref. 20). While current enzymes may be able to survive this for short periods of time, in order to be an economic process, it would be necessary to maintain this running temperature for extended periods and, to date, no enzymes with the requisite stability have been identified. However, improved thermostable GIs have been engineered from native enzymes[21,22] or isolated from natural sources[23–25]. This technology may prove adaptable to industrial processes in the future.

Conclusions

The starch-processing industry is unique within the industrial enzyme sector, in that the use of enzymes is essential for the industry. While this has resulted in greatly improved processes and products, a consequence is that the operating conditions are limited by the properties of the enzymes used. The use parameters have already been expanded by classical approaches, for example: increased production yields that result in lower costs and allow increased dosages; removal by either physical or genetic means of side activities that can lead to reversion products; and the introduction of novel enzymes such as pullulanase and thermostable AA. The demand for enzymes with improved properties and expanded use parameters to allow simpler and more robust processes persists. These future improvements will rely on accessing nature's diversity through natural isolates or protein engineering.

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Biotechnological applications of surface plasmon resonance

Vitalii Silin and Anne Plant

The surface plasmon resonance technique allows direct, real-time kinetic measurements of the interaction of unlabelled biological molecules at surfaces. After a brief discussion of the principles of surface plasmon resonance, we review its application to the nonspecific adsorption of protein, the formation of phospholipid bilayers, membrane–protein interactions and DNA hybridization. The morphological studies of the biological surfaces using surface plasmon resonance microscopy and the potential of the surface plasmon resonance to measure the dynamics of surface layer heterogeneity are discussed.

'[T]he development of a machine to monitor protein–protein and ligand–receptor interactions by using changes in surface plasmon resonance measured in real time spells the beginning of a minor revolution in biology.'[1]

The success of the surface plasmon resonance (SPR) technique for biotechnological applications is due to two basic factors: (1) SPR allows the measurement of the kinetics of biomolecular interactions in real time with a high degree of sensitivity; and (2) no labelling of the biomolecules is necessary for their detection. Ten years after the pioneering work that demonstrated the use of SPR as an optical biosensor[2], the first commercial SPR device was made available. This resulted in the rapid spread of SPR to a wide spectrum of biotechnological applications, including immunological analysis, studies of protein–protein interaction, molecular-biological studies on the mechanisms of gene expression, signal transduction and cell–cell interactions, the screening of new ligands, the quantification of protein adsorption and immobilization, the evaluation of surfaces for biocompatibility, epitope mapping, the determination of affinity constants, and the examination of the kinetics of binding (for reviews see Refs 3–5 and http://www.biacore.com/scientific/index.html). In this article, recent work on nonspecific protein adsorption, phospholipid-vesicle adsorption, fusion of phospholipid vesicles with a surface and DNA hybridization will be discussed in light of the continuing development of the SPR technique.

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Surface plasmon resonance

Plasmons, or plasma waves, are charge-density oscillations propagating in a plasma, for example a 'gas' of charged particles; in the case of a metallic solid, the plasma consists of a 'gas' of free electrons. Plasma oscillations that can propagate at the interface of a metal and a dielectric medium are called surface plasmons (SPs). For SPs to exist at an interface, such as a metal–water interface, the two media must have dielectric permeabilities (ϵ) of opposite sign[7]. The dielectric permeability of any material is a function of the wavelength (colour) of the light, and, for any given interface, the excitation of surface plasmons will not be possible at all wavelengths. For metals, the dielectric constant (ϵ_m) is negative in the infrared-to-visible part of the spectrum; for water, the constant (ϵ_b) is positive. This allows the existence of SPs on metal–water interfaces. Gold is the most suitable metal for biotechnological SPR applications, because of its optical and chemical properties.

SPs are evanescent electromagnetic waves, with maximal intensity at the interface; the intensity decays exponentially in both the metal and the dielectric medium (for example, water) with distance from the surface. Thus the SP wave is different from a bulk wave, being specifically associated with the metal–dielectric interface. Propagation of the electromagnetic waves are characterized using so-called wave, or propagation, vectors. The propagation vectors of the SP wave and the bulk wave, k_{sp} and k_b, are defined by Eqns 1 and 2:

$$k_{sp} = k_0 \, [\epsilon_m \, \epsilon_b \, / \, (\epsilon_m + \epsilon_b)]^{1/2} \qquad (1)$$

$$k_b = k_0 \, \epsilon_b^{1/2} \qquad (2)$$

respectively, where k_0 is the propagation vector in a vacuum. The character of the SPR is associated with

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PII: S0167-7799(97)01085-8

the creation of the conditions that lead to the bulk wave being transformed to the SP wave. It requires the fulfillment of the following resonance condition: the SP propagation vector must be equal to the bulk electromagnetic wave propagation vector (Eqn 3).

$$k_{sp} = k_b \quad (3)$$

One way in which the resonance condition can be achieved is by using prism-couplers with refractive indices, n_p, larger than $\epsilon_b^{1/2}$, where $n_p = \epsilon_p^{1/2}$ (where ϵ_p is the dielectric constant of the glass prism).

Two of the optical configurations[7] for SP excitation are the Otto configuration and the Kretschmann configuration. The Kretschmann configuration, which is shown in Fig. 1, is the most suitable for biotechnological SPR applications. In this configuration, plasmons are excited at the surface of a gold film deposited on a prism. The thickness of the gold film is critical – the optimal thickness, d, of the gold film depends on the optical constants of the boundary media and on the wavelength, λ, of light. For a gold film, the optimal d is 45 nm at λ = 790 nm. The efficiency of conversion of bulk waves into SPs decreases with increasing thickness and decreasing transparency of the gold film. If the film is too thin, however, rapid reconversion of SP waves into bulk waves through the prism significantly changes the width of the resonance curve, reducing the sensitivity of the SPR measurements.

Resonance is a function of the angle of incidence, φ, in the following way (Fig. 1; Eqn 4):

$$k_{sp} = n_p\, k_b \sin\varphi \quad (4)$$

A sharp minimum in the reflection coefficient is observed at a particular angle of the incident light (Fig. 1). This minimum is the result of maximal coupling of the incident light into SPs, which results in maximal absorption of the incident light by the gold film. The angle at which the SPR minimum occurs is strongly dependent on the refractive indices of all the boundary media, including the gold film, the bulk solution in contact with the gold-coated prism and additional layers such as molecules deposited on the surface by adsorption from solution. For a multilayered surface, the description of k_{sp} becomes much more complicated than Eqn 1[7]. In general, k_{sp} increases with increasing biolayer thickness at the gold surface, resulting in increases in the resonance angle (Eqn 4). The thicker the biolayer on the gold surface, the greater the SPR minimum shifts to higher angles; this shifting can be measured in real time during the process of adsorption of biomolecules at the surface. The SPR technique is extremely sensitive to optical thickness changes, and a resolution of better than 0.1 nm can be achieved.

Lasers or LEDs are used as light sources for SPR measurements. A distribution of angles of incidence is created using a cylindrical lens that focuses the incoming light on the prism base (Fig. 2). Modern SPR devices use linear arrays of charge-coupled devices (CCDs) to detect reflected light from the surface. The use of array detectors allows the measurement of light reflected at a wide range of angles (>3°) and avoids the need for mechanically controlling the angular position of the detector. Devices without CCD detectors are limited to fixed-angle measurements at a high time resolution. The fixed-angle measurements can lead to complications in signal analysis caused by SPR line broadening and, therefore, introduce significant errors into the interpretation of the kinetic data.

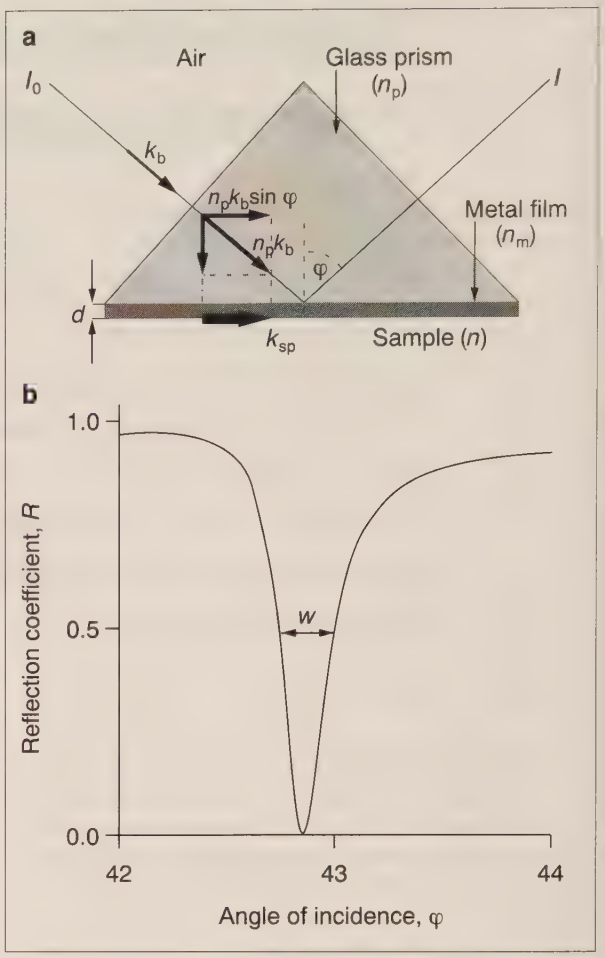

Figure 1

Surface plasmon resonance (SPR) in theory. (**a**) Diagram of surface plasmon excitation. The bulk propagation vector, k_b, increases n_p times inside a prism with refractive index n_p. Changing the angle of incidence, φ, the component of the propagation vector parallel to surface ($n_p\, k_b \sin\varphi$) matches the surface plasmon wave vector k_{sp} on a metal surface with a refractive index n_m and thickness d. A refractive index of n corresponds to water. I_0 and I are the intensities of the incident and reflected laser beam, respectively, and $R = I / I_0$ is the reflection coefficient. (**b**) Dependence of the reflection coefficient on the angle of incidence. The sharp minimum appears due to SP excitation. The SPR line width is indicated by w.

The detection limit of modern SPR devices is better than 0.5 ng cm^{-2} surface concentration, 0.1 nm film thickness or 10^{-12} M analyte concentration. To carry out experiments close to the detection limit, the samples, buffers and protein solutions must be properly prepared. Any inhomogeneity, impurities, gas

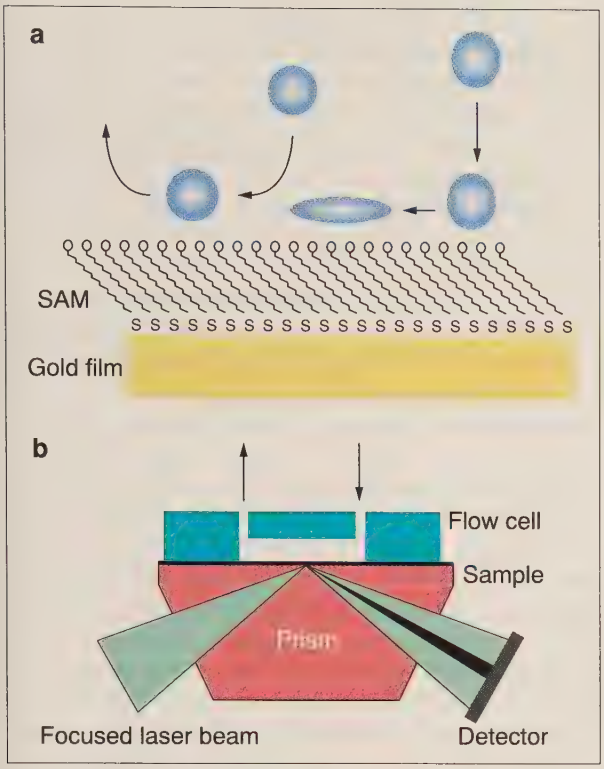

Figure 2
A schematic diagram of the surface plasmon resonance technique applied to the detection of biomolecule adsorption. (**a**) An enhanced view of the gold sample surface. Biomolecules from the solution can adsorb reversibly or irreversibly at the surface and can change conformation during this interaction. (**b**) Biomolecule adsorption is observed by the use of a light source and CCD detector, viewing through a glass prism and a glass substrate covered with thin gold film (~50 nm) modified with a self-assembled monolayer, and using a flow cell to deposit the adsorbent, causing the biomolecule solution to flow over the sample surface as indicated by the arrows. The converging beam and CCD detector allow measurements to be taken at a wide range of angles simultaneously. The dark portion of the reflected beam appears due to the surface-plasmon excitation, its position in the detector depending upon the amount of adsorbed material on the sample.

bubbles or temperature fluctuation will lead to increases in the noise. The sensitivity to analyte concentration depends strongly on the affinity constants and specificity of the immobilized biomolecules on the surface, their surface concentration, packing density, orientation and denaturation level.

SPR measurements can be carried out under either continuous-flow or nonflowing conditions. In general, very small volume (μl) flow cells are used to minimize the consumption of material. The use of flow cells is preferred because they minimize irreproducibility in the mixing of solutions added to the cell, control the diffusion-layer thickness and reduce changes in concentrations in the cell due to adsorption of material onto the cell walls.

The experimental procedures for SPR measurements include several other aspects not mentioned here, including modification of the gold surface to form specific active sites on the surface, real-time detection of the surface interaction and the use of mathematical analysis to extract the kinetic constants of the biochemical reaction. For details on the SPR technique, flow-cell characterization, and kinetic-curve analysis, see Ref. 8.

Kinetics of biomolecular interactions at a surface

Typically, SPR data indicate changes in the concentration of a surface species or the thickness of a surface layer as function of time. Figure 3 shows the application of SPR to a study of nonspecific protein adsorption[9] to gold surfaces modified by alkylthiol self-assembled monolayers[10] (SAMs). The reduction of nonspecific adsorption is an important goal in the efforts to improve the sensitivity and specificity of biosensors. The nonspecific binding of human immunoglobulin G (hIgG) and bovine serum albumin (BSA) was studied on SAMs with the following terminal groups: CH_3, C_6H_5OH, COO^-, NH_2^+, OH and oligoethylene oxide (OEO). The ability of the SAMs to adsorb hIgG decreased in the following order: CH_3 > C_6H_5OH > COO^- > NH_2^+ > OH > OEO; their ability to adsorb BSA, however, decreased in the following order: C_6H_5OH > CH_3 > COO^- > NH_2^+ > OH > OEO. These results also indicated that on the OEO SAM, the surface concentration of these proteins was less than 0.5 ng cm^{-2} (the detection limit of the SPR device). This was approximately 10^{-3} times the binding that occurred on the hydrophobic CH_3-terminated SAM surfaces. The kinetics of the binding curves for the adsorption of proteins was described in terms of multiple states of adsorbed proteins that involve multipoint hydrophobic, electrostatic and hydrogen-bond interactions for the different surfaces, and protein lateral interactions caused by the unfolding of adsorbed proteins[9].

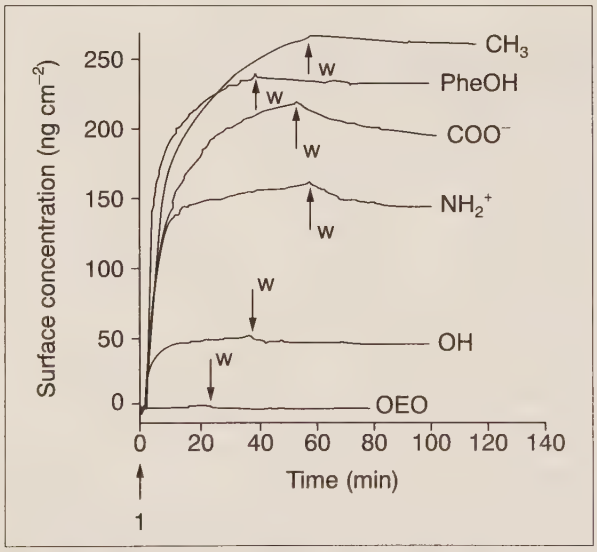

Figure 3
Kinetics of human immunoglobulin G (hIgG) adsorption on gold surfaces modified with CH_3, PheOH, COO^-, NH_2^+, OH and OEO SAMs. Arrow 1 shows the time when the protein-solution flow was started (hIgG concentration = 0.5 μM); the arrows marked 'w' indicate the time when washing was begun.

Supported hybrid-bilayer membranes (HBMs), composed of a monolayer of phospholipid (PL) and a SAM associated with a metal surface, are useful as model lipid-bilayer membranes for studying membrane-receptor–ligand and cell–cell binding events, and as a method for protein immobilization onto the surface for biosensor construction. SPR is a useful technique for studying the formation of stable PL layers on solid supports and the incorporation of membrane proteins into such layers, either for the development of highly selective biosensors based on molecular recognition or for the immobilization of biomolecules for kinetic studies.

Studies of specific binding of neutravidinylated and biotinylated lipid vesicles to well-defined HBMs have been performed under conditions of continuous flow using a commercial SPR instrument[11] (Fig. 4). HBMs composed of a monolayer of PL and a monolayer of alkylthiol were shown to be stable in flow and to block the nonspecific adsorption of proteins including BSA, IgG and wheatgerm agglutinin. The use of HBMs in flow provides a means of conducting equilibrium and kinetic studies of ligand–receptor interactions with receptors or ligands in a cell-membrane-like environment.

SPR data were used to determine the optical parameters and thicknesses of PL layers on bare silver surfaces[12,13] formed using the Langmuir–Blodgett technique. Formation of a PL bilayer by fusion of PL vesicles with an amphiphilic copolymer film adsorbed to a gold surface was observed by SPR[14], and it was also shown that bilayers can be formed from negatively charged and biotinylated PL vesicles, and subsequent specific binding of avidin to the biotinylated PL layer was observed.

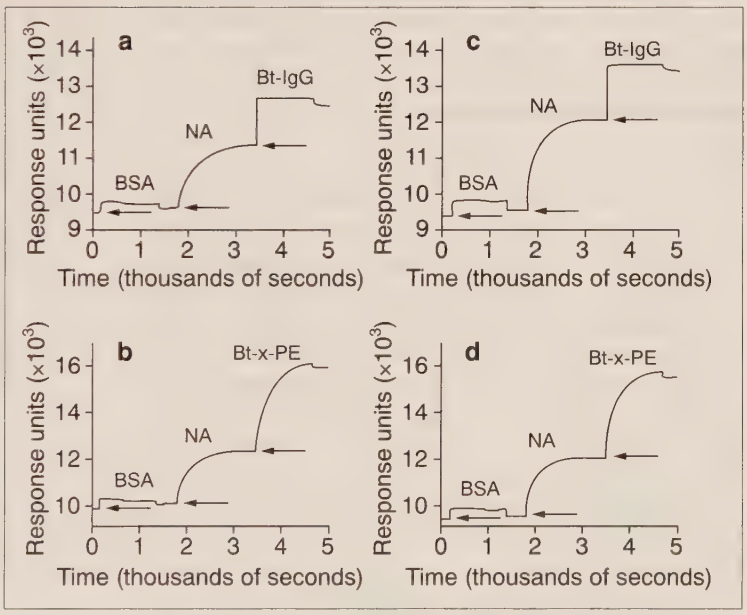

Figure 4

Surface plasmon resonance response of hybrid bilayer membranes containing 2% (**a**,**b**) or 10% (**c**,**d**) N-[(6-biotinyl)amino]hexanoyl-1,2-hexadecanoyl-sn-glycerophosphoethanolamine (Bt-x-PE). Arrows indicate the time of sample injection. The injection volume was 40 μl. The Y axis, measured in response units (RU), is directly proportional to the change in the SPR resonance angle, which is sensitive to the thickness of the layer at the gold surface. Bovine serum albumin (BSA; 0.5 mg ml^{-1}; 2 injections), neutravidin (NA; 125 μg ml^{-1}), biotinylated immunoglobulin G (Bt–IgG; 0.5 mg ml^{-1}) and 1-palmithiol, 2-oleoyl phosphatidylcholine vesicles containing 10% Bt-x-PE (20 μM total lipid) were injected as indicated.

Pentapeptides have been used as spacers to provide a hydrated region separating the solid-supported PL layers from the gold substrate to allow the incorporation of transmembrane proteins[15]. SPR has been used to monitor the coupling of phosphatidylethanolamine to the carboxyl groups of surface-bound peptides in *in-situ* SPR, and capacitance measurements were used to determine the bilayer thickness when the surface was exposed to a suspension of liposomes. An active transmembrane protein, ATPase, was incorporated into the bilayer, and proton pumping demonstrated.

The kinetics of the formation of a phospholipid (egg-lecitin) layer on the mixed SAM layer containing hydrated spacers was studied by SPR[16]. A mixed SAM layer was formed by varying the proportion of cholesterol, modifying it with hydroxyethyleneoxythiol and a packing molecule, giving a hydroxyethyleneoxy SAM. The results showed that a lipid bilayer is added to the hydrophilic component of the hydroxyethyleneoxy SAM and a lipid monolayer to the hydrophobic component, octadecanethiol SAM. In the mixed SAM, the cholesterol moiety inserts itself into the bilayer, and the thickness of the lipid layer falls between that of the monolayer and the bilayer. No adsorption of intact liposomes was observed.

The addition of a PL bilayer onto a negatively charged, COO^--terminated surface from negatively charged PL vesicles has been followed by SPR[17]. This process occurred via Ca^{2+} ion bridging, and so rinsing with a buffer without Ca^{2+} ions resulted in the loss of the bilayer. The authors claim that this fusion is the result of electrostatic and 'hydration' interactions. Comparison of SPR, ellipsometric and capacitance data indicated differences between ellipsometry and SPR measurements, but coincidence between SPR and capacitance data.

SPR and impedance measurements were applied to the study of the interaction of cholera toxin with a PL monolayer containing monosialoganglioside (G_{M1})[18], deposited from a detergent solution onto a gold surface modified with a hydrophobic SAM. The two techniques provided different but complementary information. An increase in capacitance accompanied increasing concentration of G_{M1} in the PL monolayer. A corresponding increase in the amount of cholera toxin bound to the surface was found by SPR. SPR indicated that the thickness of the lipid layer did not change with changes in the G_{M1} concentration in the absence of toxin, and the capacitance did not change as a function of cholera-toxin binding. SPR studies of the cholera-toxin binding affinity and specificity for gangliosides have also been reported[19].

DNA hybridization

The ability of the SPR technique to monitor biomolecular interactions directly and in real time has been applied to the problem of DNA hybridization.

Glossary

BSA	Bovine serum albumin
CCD	Charge-coupled device
HBM	Hybrid bilayer membrane
LED	Light-emitting diode
OEO	Oligoethylene oxide [11-mercaptoundecyl hexa(ethylene oxide)]
PL	Phospholipid
SAM	Self-assembled monolayer
SP	Surface plasmon
SPM	Surface plasmon microscopy
SPR	Surface plasmon resonance
d	Metal (gold) film thickness
ϵ_b	Dielectric constant of bulk media
ϵ_m	Dielectric constant of metal film
ϵ_p	Dielectric constant of glass prism
I	Intensity of reflected beam
I_0	Intensity of the incident beam
k_b	Propagation vector of the light in bulk media
k_0	Propagation vector of the light in vacuum
k_{sp}	Propagation vector of the surface plasmon at the surface of the metal
n	Refractive index of the biomaterial solution
n_m	Refractive index of the metal film
n_p	Refractive index of the glass prism
φ	Angle of incidence
R	Reflection coefficient
w	Width of the surface plasmon resonance curve

The first reports[20–22] showed the feasibility of using SPR for this purpose and, more recently[23,24], the use of SPR for DNA hybridization has been studied in greater detail. In both cases, the immobilization of DNA fragments onto the SPR sample surface was carried out using the high-affinity streptavidin–biotin interaction. The SPR has been applied for the detection of DNA strand separation, hybridization kinetics and enzymatic modification[23]. In this work, SPR was used to monitor all the steps in the manipulation of the DNA at the surface, including the immobilization of the biotinylated DNA on the sample surface, its hybridization with the complementary oligonucleotide, the use of DNA ligase to attach an additional fragment of DNA covalently to the first fragment, and a second hybridization reaction. As a result of real-time monitoring, it was concluded that no nonspecific interaction with the surface took place during the experiment. Also, noncomplementary fragments did not bind to DNA at the surface. The kinetics of hybridization allowed the identification of degrees of strand complementarity.

SPR has been used to study the effect of single-base-pair mismatches on DNA hybridization kinetics[24]. Association and dissociation kinetics were determined for the hybridization of unlabelled DNA, and significant destabilization was observed, due to a single-base-pair mismatch in the duplex formed between an immobilized 20mer oligonucleotide probe and 11-, 13- and 15mer target oligonucleotides. The association kinetics for DNA hybridization was observed to be inversely proportional to the number of base pairs mismatched; the dissociation kinetics was even more strongly influenced by mismatches. The authors claimed that this strategy may lead to the use of SPR for mutation detection in the diagnosis of genetic disease and various forms of cancer.

SPR microscopy

Due to its remarkable sensitivity to surface-layer thickness, SPR can be used for surface morphological investigations of thin biomolecular films by microscopic imaging. SPR imaging can determine the amount and distribution of adsorbed molecules at a surface, and can provide these answers in real time with high temporal resolution [for example, two-dimensional SPR microscopy (SPM) has been used to image potential waves in an electrochemical system with a temporal resolution of up to 800 frames per sec[25]]. The invention of SPR microscopy[26–28] led to the first application of SPR in the imaging of biomolecular surface structures[29]. SPM images can be obtained using the experimental configuration shown in Fig. 2 by using a parallel beam[27,28] for uniform illumination of a surface at a fixed angle. Surface inhomogeneities in thickness or refractive index result in differences in the intensity of the reflected beam forming the image at the CCD camera. This configuration is sufficient to determine thickness with Ångstrom-level resolution, but the lateral resolution is much less impressive (~5 μm). A comparison of SPM and fluorescent microscopy was carried out on dimyristoylphosphatidic acid monolayers, which form quasi-two-dimensional structures[29]. It was shown that SPM permits the real-time imaging of the growth of solid crystalline domains within the fluid phase, without requiring the use of fluorescent labels. In addition, SPM enables a quantitative analysis of the optical properties of the coexisting domains, despite the fact that it provides a lower lateral resolution than fluorescent microscopy.

SPM has been applied to the study of the formation of structured, supported lipid bilayers for the reconstitution of transmembrane proteins[30]. The generation of the pattern of two types of region on a gold surface with covalently bound thiolipid and free palmitic acid was observed and analysed using SPM; a domain structure of PL was found at the gold surface covered with a mixture of thiolipids and thiols and the mixed hydrophilic–hydrophobic SAM–thiolipid surface. For the thiolipid–carboxythiol domains, it was shown that wetting of the surface significantly enhanced the contrast of the SPM image.

SPM has also been used for the detection of structural defects in samples containing biotinylated mercaptans, in a study of the recognition of surface biotin by streptavidin[31], and also in the detection of the domain structure for the binary mixture of PL modified with biotin and PL without biotin[32]. SPM has been used to probe the formation of bilayers of painted supported lipid membranes at a gold surface by applying ±100 mV rectangular potential pulses between the gold film and a gold electrode in the electrolyte solution; these pulses promote PL bilayer formation[33].

It has been shown that the SPM may be applicable to the study of oligonucleotide hybridization, as the image contrast for two oligonucleotides at a surface, one fully homologous to the target sequence and the other not, is large[34]. This result provides a basis for the development of multiple-array detection schemes for the massive parallel processing of oligonucleotide hybridization with the use of SPM.

Dynamics of lateral heterogeneity in biomolecular layers

The major limitation associated with SPM is the poor lateral resolution, which restricts the application of this method to systems that exhibit relatively large features. The SP response, however, is sensitive to surface heterogeneities on a much smaller scale. It has been shown that during protein adsorption to a modified metal surface, not only does the SPR minimum shift, but changes also occur in the SPR line width, *w*, (Fig. 1b) and in the depth of the SPR minimum[35]. We assume that changes in SPR line width result from molecular-scale inhomogeneities of the biolayer that arise during protein adsorption, including island formation, formation of the second layer and changes in the bilayer packing density, biomolecule orientation and denaturation. SPR line broadening was monitored in real time during the adsorption of a hIgG layer onto methyl- and OEO-terminated SAMs[9]. In the case of the OEO surface, which resists protein adsorption[9] (Fig. 5), no changes in the SPR line width were observed. For the methyl-terminated SAM surface, on which protein adsorption occurred readily, the line width changed with both time and the amount of protein at the surface. Increases in surface molecular roughness, determined by increasing SPR line width, paralleled the initial rapid adsorption of protein. As adsorption progressed further, with a slow increase in the apparent thickness of the layer, the SPR line width decreased, suggesting that changes were occurring in the surface morphology. This response could be due to a slow increase in the packing density of protein at the surface, resulting in more homogeneous surface coverage. SPR line broadening may be useful in providing information concerning the spatial heterogeneity of biomolecular interactions at the surface as well as morphological changes during adsorption. The spatial sensitivity and the details associated with interpretation of SPR line broadening have yet to be determined.

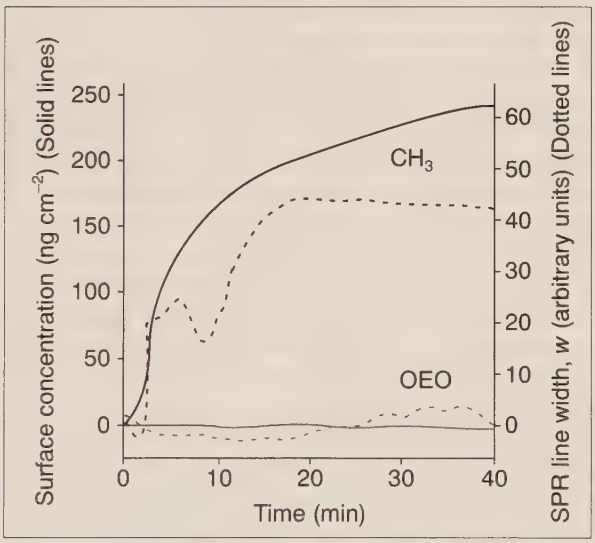

Figure 5

The increase in the surface plasmon resonance line width, *w* (Fig. 1b), with time (dotted lines) due to adsorption of human immunoglobulin G on methyl- and oligoethylene-oxide-terminated self-assembled monolayer surfaces. The solid lines are kinetic curves for these surfaces. Measurements of the surface plasmon resonance shift and width were carried out simultaneously.

Conclusion

The sensitive, label-free and fast-response SPR technique is an extremely suitable tool for solving a wide range of biotechnological problems. SPR can be used for all steps in the preparation of a surface for biotechnological measurements, starting from modification of the bare gold, through biomolecule immobilization to various kinds of surface manipulations that result in the required surface concentration, biomolecule conformation or bilayer morphology. The necessity of using a metal surface for SPR excitation and the low sensitivity to low molecular weight species are major limitations of the SPR method. The first limitation can be overcome by depositing a thin film of the material of interest onto the gold surface. The second is more difficult, but in those cases where the surface concentration of small molecules is large, as with SAM layers, the kinetics of layer formation can be detected.

SPR is a versatile technique that can be used in combination with electrochemical measurements[25], HPLC[36] and atomic force microscopy[37]. SPR can also be used as an imaging technique (e.g. SPM). Although the SPR technique is still primarily a research tool, the development of new commercially available SPR devices for specific medical applications[38] will permit routine experimentation with this technique in clinical as well as scientific laboratories.

New applications of SPR are constantly being developed. We have recently studied the kinetics and compositional analysis of a red-blood-cell membrane–alkanethiol hybrid bilayer (Rao, N. M. *et al.*, unpublished). We are currently pursuing the analysis of SPR line broadening to examine spatial heterogeneity in biomolecular surfaces, as well as the use of SPR to enhance Raman spectroscopy of biological molecules at surfaces in order to provide information on molecular conformation as well as layer structure. We believe that these developments, together with SPM, will significantly enhance the potential of SPR for future biotechnological applications.

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Cold-adapted enzymes

Craig J. Marshall

It is an article of faith among biochemists and molecular biologists that precious enzymes must be stored on ice. The usual reason given is that, at temperatures around freezing, enzyme activity is minimized and protein stability maximized. There is considerable evidence supporting this, but is it true for all enzymes? What about enzymes from organisms that spend part or all of their lives at temperatures around freezing? How do they manage to maintain normal enzymatic function at low temperatures? Can we learn something from cold-adapted proteins that would allow us better to understand how proteins function?

If the rates of reactions mediated by temperate enzymes are measured at a number of temperatures and extrapolated to low temperatures, it is found that at about 0°C, the reaction rate falls to zero[1]. A number of factors probably contribute to this reduction of reaction rate with temperature, including changes in pH and lowered rates of diffusion of substrate and product. Perhaps the most important factor is a reduction of the catalytic efficiency of the enzyme caused by a decrease in the ability of the enzyme to change shape or 'breathe' during the process of catalysis.

However, there are many organisms that normally live at temperatures about or below freezing and yet maintain their normal metabolic processes. How are proteins from these cold-adapted organisms modified to function efficiently at these temperatures?

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The effects of cold on aqueous systems

The viscosity of water increases severalfold as the temperature decreases from 20°C to 0°C; the rates of diffusion of both solute and water molecules are reduced as the temperature falls; the solubility of salts

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PII: S0167-7799(97)01086-X

declines with temperature, while that of gases increases; the pH of biological buffers (typically based on carbonate) shows a significant increase with decreasing temperature, which may well have an effect on protein function[2,3], affecting both solubility of proteins and the charge on amino acids, particularly histidine residues (pK≈6.5–7.4); most importantly, the rate of reaction falls in an exponential fashion, so that a fall of 10°C is typically associated with a twofold decrease in reaction rate, defined as a Q_{10} of approximately two (Box 1)[4].

Why should a small difference in the total energy of molecules involved be associated with such a large change in reaction rate? The answer lies in the formation of an activated complex. The energy of activation, E_a, is a measure of the energy required to form an activated complex and is very temperature dependent. Catalysts in general, and enzymes in particular, affect the kinetics of reaction by lowering E_a, allowing more substrate molecules to form an activated complex. We might expect cold-adapted enzymes to be modified to counter the expected increase in E_a as temperatures decrease.

The study of enzymes from organisms adapted to extreme environments has a long history and has told us something of the general patterns of protein structure and function. Thermophilic organisms have attracted much attention for this reason, as well as because of the potential industrial uses of enzymes that function at high temperatures[5–7]. The study of cold-adapted proteins also has the potential both for understanding enzyme function and for novel industrial applications[1,8].

Cold denaturation of enzymes

A number of enzymes are susceptible to a phenomenon known as cold denaturation, in which a reversible alteration of protein structure is responsible for the loss of enzyme activity at low temperatures[9].

Box 1. The Arrhenius equations

The temperature dependence of biological reaction rates is often interpreted in the manner suggested by Arrhenius at the end of the last century. The relationships defined by Arrhenius,

$$k = Ae^{-E_a/RT}$$

$$\ln k = \ln A - E_a/RT$$

where k is reaction rate and A is a 'pre-exponential' factor that allows the determination of the activation energy, E_a, from the slope obtained from a plot of $\ln k$ against $1/T$ (Ref. 4).

The term Q_{10} is related to E_a, and is often used to describe the sensitivity of reaction rate to temperature. A reaction with a Q_{10} of 2 has a reaction rate that doubles with a 10°C increase in temperature. Reactions with $Q_{10} < 2$ are likely to be diffusion-limited whereas those $Q_{10} > 2$ probably involve entropy effects associated with structural rearrangements.

Among the several dozen enzymes known to be affected by cold denaturation are a number of ATPases and fatty-acid synthetases; they are typically found to be multimeric. Cold denaturation is usually reversible and is often, although not universally, characterized by dissociation of the quaternary structure.

Cold denaturation is thought to result from interactions between nonpolar groups in proteins and water, which become thermodynamically favoured at low temperatures. The breakdown of hydrophobic forces that are important in protein folding results in unfolding of the protein at low temperatures[9]. Clearly, enzymes from cold-adapted organisms must be resistant to cold denaturation; from what is known of cold denaturation, it seems likely that hydrophobic forces might be less important in cold-adapted enzymes.

Enzyme function

The overall catalytic cycle of an enzyme can be divided simplistically into three main phases: recognition and binding of substrate, mediated by a number of amino acids within the enzyme; conformational stresses induced by the changes in the active site, leading to the formation of product; release of product from the enzyme. Each of these phases involves weak bonding interactions that are sensitive to temperature[5,10–12]. For the enzyme to be returned to a state where it can participate in the catalytic cycle, each of these steps must be completed efficiently.

Efficient binding of substrate by the enzyme is mediated by a number of comparatively weak forces, among which are hydrogen bonds and hydrophobic interactions. At low temperatures, the binding of substrate is likely to be affected, because diffusion is slowed down and the viscosity of the fluids increased, but, once bound, the substrate may well be less likely to be displaced from the enzyme, because of reduced thermal motion that results from low temperature. This may help to stabilize the enzyme–substrate complex and inhibit the formation of the transition state required for catalysis to occur.

The aspect of enzyme function most likely to be affected by low temperature is the catalytic cycle. Enzyme catalysis is characterized by structural changes in both the protein and the substrate. Sometimes, these changes in the enzyme are subtle, involving just a few amino acid side chains, but the enzyme movement may be on a far larger scale, with whole domains moving with respect to one another[13]. The formation of a transition state in most enzymes involves some degree of strain being imposed on the substrate. A reduction in the ability of the enzyme to undergo structural changes will affect the rate at which the transition state can be produced. The capacity of enzymes to undergo such structural movements will be reduced at low temperatures, as a consequence of reduced thermal motion[1,11,14] (Table 1).

Product release is potentially important in cold-adapted enzymes. If products remain bound to the enzyme, a new catalytic cycle cannot be started. Retention of products is likely to be enhanced at lower temperatures

Table 1. Temperature sensitivity of enzyme properties[10,11]

Protein property	Probable temperature sensitivity
Primary structure	minimal
Secondary structure	sensitive
Tertiary structure	sensitive
Quaternary structure	very sensitive
Membrane interactions	very sensitive
Substrate or product stability	possibly sensitive
Ligand binding	very sensitive
Catalytic function	very sensitive
Regulation	very sensitive

for the same reasons that substrates might be expected to bind more tightly to enzymes. It is important to note that tight binding of either substrate or product does not necessarily lead to rapid enzyme action.

How might proteins be cold-adapted?

If enzymes are cold-adapted, what form might this adaptation take? A number of studies have shown that the fundamental kinetic properties of enzymes are conserved from species to species within the normal physiological range of temperatures encountered by that species. In this context, we are considering organisms whose body temperature matches that of the environment in which they are found and not animals, such as mammals, that maintain body temperature more or less independently of the ambient temperature. A graphical depiction of conservation of kinetic parameters is shown in Fig. 1. The value of the kinetic parameters (e.g. k_{cat} or K_m) of each individual enzyme increases with temperature, and is bounded at the upper limit of temperature by thermal denaturation of the protein and at the lower limit by the reduction of enzyme activity to zero or by cold denaturation. If temperature-dependent kinetic data from homologous enzymes of organisms with different physiological temperature ranges are plotted on the same graph, they produce a series of approximately parallel lines. The kinetic curves of enzymes from animals from warm climates tend to be shifted to the right, and those from animals of cold climates to the left. This results in conservation of the kinetic parameter at the temperature at which the organism usually lives. For example, enzymes from Antarctic fish have higher kinetic parameters at −1.8°C than do enzymes from similar temperate fish if measured at −1.8°C. However, if the kinetic parameters of enzymes from temperate fish are measured at their physiological temperature, they have the same values as those of Antarctic fish at their respective physiological temperature[14,15].

A number of cold-adapted proteins have been examined to see if this generalized finding holds true for proteins from cold-adapted organisms. The majority of the cold-adapted organisms examined have been either fish or bacteria from cold Antarctic waters, but

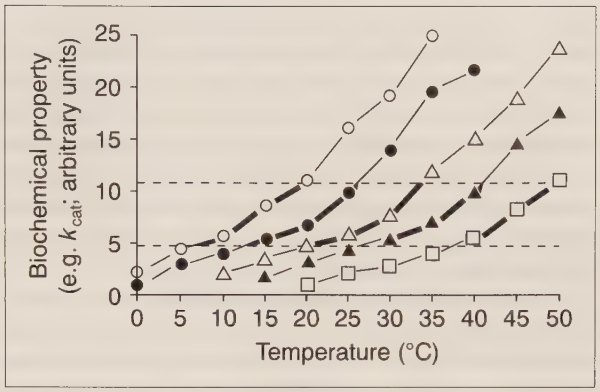

Figure 1

A generalized model showing how the biochemical properties of organisms, such as k_{cat} or K_m, may be conserved within narrow temperature ranges corresponding to the physiological temperature ranges of the species concerned. The five curves illustrate the temperature dependence of a biochemical or kinetic property common to five species with a different range of body temperatures. The darker part of each curve represents the normal range of body temperature and shows how the biochemical or kinetic property retains a similar value from species to species. For example, the normal range of the most cold-adapted species (open circles) is 6–20°C, while that of the most thermophilic species (open squares) is 38–48°C. At physiological temperatures, the biochemical parameter is conserved within a defined range, in this case between five and ten arbitrary units. Reproduced with permission from Ref. 14.

some Arctic organisms have also been examined (Table 2). Other than modelling studies based on the sequences of these enzymes, the only structural information comes from *Alteromonas haloplanctis* α-amylase[16], which has been crystallized and the structure partially solved[17].

In addition to bacterial enzymes, considerable work has been performed on proteins from cold-adapted fish. Most of this work has concentrated on Antarctic fish, which are exposed to a temperature of about −1.8°C (the freezing point of seawater) for most of the year[18], although enzymes from the cold-adapted Atlantic cod *Gadus morhua* have also received considerable attention. Antarctic fish are probably the most cold-adapted vertebrates, as their body temperature is below the expected freezing point of the body fluids for most or all of the year[19] and so enzymes from these fish have attracted much interest (Table 2). Freezing of body fluids in these fish is prevented by 'antifreeze' glycopeptides and proteins that inhibit ice-crystal growth. Similar antifreeze molecules are found in both Arctic and Antarctic fish, although they appear to have different evolutionary origins[20,21].

Although, strictly speaking, microtubules are not enzymes, the rates of polymerization and depolymerization of these proteins are temperature sensitive. Microtubules from both Arctic and Antarctic cold adapted fish show adaptation to the cold and remain polymerized at temperatures far below those that cause depolymerization of microtubules from temperate fish[34,40].

Do these studies support the notion that enzymes are modified so that kinetic parameters of enzymes are

conserved at low temperatures? At any given temperature, cold-adapted enzymes tend to have a higher k_{cat} and K_m than is found for their temperate homologues[1]. Furthermore, broadly speaking, these parameters are found to be conserved at physiologically relevant temperatures, as our model predicted. Although it seems clear that kinetic parameters are conserved, little is known of the structural basis of this conservation and the question remains open

The structure of a cold-adapted haemoglobin from the Antarctic fish *Trematomus bernacchii* has been solved in both the R and T forms[41,42] (states of high and low affinity for oxygen, respectively, associated with structural changes in the protein), and was found to be very similar to other fish haemoglobins. Haemoglobin may not show the cold-adaptation discussed above, because the increased viscosity of blood at −1.8°C and the increased solubility of oxygen and carbon dioxide have resulted in a reduced role for haemoglobin in many Antarctic fish. This process is carried to an extreme in icefish, which have no myoglobin or haemoglobin, although the remnants of genes for these proteins are present[43]. Other Antarctic fish are resistant to carbon-monoxide poisoning, suggesting that, even without haemoglobin, sufficient oxygen can be carried by the blood to meet resting metabolic needs[44].

What is the structural basis of cold-adaptation?

Amino acid sequence data has allowed comparative modelling studies of some cold-adapted enzymes[6,8,16,25,27,29,38] and some interesting general trends have emerged. Cold-adapted enzymes tend to have fewer salt links, reduced aromatic interactions within the hydrophobic core, reductions in the number of proline and arginine residues, a reduction in the hydrophobicity of the enzyme and an increase in the number of interactions between the enzyme surface and the solvent[1]. The introduction of one or more methionine residues in the place of other buried hydrophobic residues in the core of T4 lysozyme reduces the melting point of the resulting protein[45], suggesting this might be important in cold-adapting proteins.

These changes are all likely to increase the structural flexibility of the enzyme. It may be that a given degree of flexibility is required for enzyme function and is one of the conserved biological properties discussed above. The structural bases of cold-adaptation stand in almost direct contrast to the structural changes, such as greater hydrophobic interactions, salt bridges and hydrogen bonds, that increase the thermal stability of thermophilic proteins[12].

Although modelling studies have a limited ability to detect subtle structural changes, no significant alterations to the active site of any enzyme have been predicted in any of the models of cold-adapted proteins. This suggests that cold-adaptation does not involve new catalytic mechanisms, but that conventional mechanisms are modified to operate better at low temperatures.

Table 2. Enzymes known to show cold adaptation

Enzyme	Source	Refs
	Bacteria	
α-Amylase	*Alteromonas haloplanctis*	16,17
Isocitrate dehydrogenase	*Vibrio* species	22
β-Lactamase	*Moraxella*	23,24
Lipase	*Vibrio* species	25
	Psychrobacter immobilis	26
Subtilisin	*Bacillus* TA41	8
Triose-phosphate isomerase	*Moraxella*	27
	Fish	
Acetylcholinesterase	*Pagothenia borchgrevinki*	28
Chymotrypsin	*Gadus morhua*	29
Fatty-acid-binding protein	*Chaenocephalus aceratus*	30
Glucose-6-phosphate dehydrogenase	*Dissostichus mawsoni*	31
	Chionodraco hamatus	31
Lactate dehydrogenase	*Trematomus centronotus*	11,14,32
	Pagothenia borchgrevinki	33
Microtubules	*Notothenia gibberfrons*	34
	Notothenia coriiceps neglecta	34
Na^+–glucose cotransporter	*Trematomus bernacchii*	35
Pyruvate kinase	*Pagothenia borchgrevinki*	36
Trypsin	*Gadus morhua*	37
Trypsin	*Paranotothenia magellanica*	38,39

Thermal stability

One expected consequence of increased protein flexibility is reduced thermal stability and increased protein 'breathing'. Reduced thermal stability of cold-adapted enzymes compared with their temperate counterparts has been observed[1,10,11,14,37] and is almost universal among cold-adapted proteins. Although cold-adapted enzymes are typically more temperature sensitive than their temperate counterparts, they may be active at temperatures of up to about 45°C. Some membrane-bound enzymes show much greater temperature sensitivity[18,35,46], probably related to the breakdown of the lipid bilayer and associated proteins. Indeed, thermal breakdown of membrane proteins in the central nervous system is probably responsible for the lethal upper temperature limit of about 6°C characteristic of Antarctic fish[47]. Of particular interest is a Na^+–glucose cotransporter from the gut of the Antarctic fish *Trematomus bernacchii*. This enzyme has been characterized and found to have maximal activity at −2°C and to lose activity in an exponential fashion as the temperature rises[35].

The significance of these thermal-denaturation studies is hard to assess. Most of the methods used for determining thermal stability measure the gross unfolding of protein structure rather than reversible, thermally-induced alterations of protein function[14]. More sensitive methods of assessing thermal motion, such as circular dichroism, may reveal more information about the flexibility of protein at temperatures below that at which irreversible denaturation occurs.

Structural comparisons

It is clear that more structural information is required to understand cold adaptation in proteins better. However, without comparative data, the structure of a cold-adapted protein is unlikely to be of great assistance in understanding cold adaptation. Structural cold adaptations are likely to be subtle and may well be masked in enzymes from evolutionarily distant organisms. The chances of detecting such subtle changes may be improved by determining high-resolution structures of enzymes from organisms exposed to a range of physiological temperatures. Ideally, such an enzyme would be well-characterized with a mechanism that is understood at the structural level; in addition it is important that the enzyme has a similar role at different temperatures, to avoid differences in structure caused by changes in function rather than by cold adaptation.

To help understand specific sequence changes that lead to cold adaptation, it is important to minimize sequence differences arising from random change and to maximize differences important in cold adaptation. This requirement can be met by an appropriate choice of organisms for study. Notothenioid fish are one group of organisms that offer advantages for this type of investigation. Cold-adapted notothenioid fish dominate the fish fauna of Antarctic waters and are thought to have diverged from their temperate notothenioid relatives about 25–30 million years ago[18]. Related temperate notothenioids are found in New Zealand, South America and a number of the sub-Antarctic islands[48]. These two groups of fish provide the basis of an ideal comparative study.

It is worth keeping in mind that, as with any evolutionary character, sufficient adaptations may not be optimal adaptations.

Potential biotechnological applications

Much of the interest in thermophilic proteins came from the recognition that such enzymes were likely to have biotechnological applications. Do cold-adapted enzymes have a similar biotechnological potential? They seem likely candidates for a number of biotechnological and industrial processes in which enzymes active at lower-than-usual temperatures may be useful[1]. Several major potential applications seem possible.

- Reduced-temperature applications in which the use of cold-adapted enzymes would allow energy savings by lowering the temperature of a reaction without sacrificing enzyme activity. For example, detergent replacements (lipases and proteases) to allow colder wash cycles and low-temperature digestion of industrial wastes.
- Enzymes involved in the food industry, in which the use of cold-adapted enzymes would allow product modification at temperatures where microbial growth is minimized.
- Environmental bioremediations at low temperatures.
- Biotechnological applications: where enzyme activity at low temperature is an advantage (e.g. where substrate or product stability is enhanced); where the enzyme can be denatured at comparatively low temperatures.

Two approaches may be taken to finding biotechnologically useful cold-adapted enzymes. The first involves the screening of naturally occurring cold-adapted enzymes for useful properties. Psychrophilic bacteria are likely to be a useful resource for this work, although it suffers from the drawback that it may take much effort to characterize new enzymes adequately. Furthermore, this approach sometimes yields enzymes with interesting properties but no obvious application.

An alternative strategy is to modify existing enzymes to function at lower temperatures. Such a strategy requires a much better understanding of the nature of cold-adaptation, but has the great advantage that a well-characterized enzyme of known application is likely to result. Once more is known of the bases for cold-adaptation, this approach is likely to prove fruitful.

Acknowledgements

The support of the Marsden Fund of New Zealand is gratefully acknowledged.

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Polymeric controlled delivery for immunization

Rebecca M. Kuntz and W. Mark Saltzman

Current vaccine technology has limitations; for example, most vaccines require repeat administration for long-term protection, and immunity at mucosal surfaces is difficult to achieve. In animal models, polymeric controlled-release systems provide long-lasting systemic or mucosal immunoprotection, often after a single administration. Polymeric devices that deliver a controlled amount of antibody can provide passive immunity against genital herpes infections in mice; orally administered polymeric-microsphere-based vaccines produce enhanced immune responses in rodents and primates. These new delivery technologies have many desirable features, and so their use in humans could have a substantial impact on worldwide public health.

Approximately 200 years ago, Jenner presented his findings that inoculation with cowpox material provided protection from smallpox infection[1]. This procedure, called vaccination by Pasteur[2], eventually led to the eradication of smallpox. Similar immunization strategies have protected countless individuals from infectious diseases. Protective vaccines can be composed of live-attenuated or inactivated pathogens, purified or synthetic antigenic material, live viral vectors, preformed antibodies, or naked DNA. When antigenic material is introduced into the body, a procedure called active immunization, the immune system responds by generating molecular and cellular protection against the specific pathogen. In passive immunization, on the other hand, antibodies that neutralize a specific pathogen are administered to elicit protection with no stimulation of the host immune system.

Modern vaccine technology, which relies primarily on injected doses, can efficiently stimulate systemic immunity for some pathogens, although repeated 'booster' injections are usually required. Mucosal immune responses, particularly those leading to the secretion of antibodies in mucus, are more difficult to elicit. Mucosal immunity is important in blocking the

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PII: S0167-7799(97)01087-1

transmission of sexually transmitted diseases (STDs); stimulation of immunity at the reproductive mucosa could decrease the likelihood of transmission of diseases such as genital herpes, chlamydial infection and, possibly, AIDS. Oral vaccination can sometimes stimulate mucosal and systemic immunity, as in the case of oral polio vaccine[3]. Orally induced mucosal immunity often leads to antibody production at distant mucosal sites[4], but the orally administered antigen must survive the acidic environment of the stomach and reach immune tissue in the Peyer's patches of the gut. Providing a barrier between the vaccine and the stomach fluids should increase retention of antigenicity, while targeting the antigen to Peyer's patches should enhance stimulation of the intestinal mucosa.

Polymers play an integral role in biotechnology, with applications ranging from surgical sutures through heart valve replacements to drug-delivery vehicles. In the past decade, polymers have been designed to reduce the need for reimmunization and to target vaccine molecules to specific tissue sites for maximal action. The controlled release of macromolecules from polymeric devices was first demonstrated in 1976 by Langer and Folkman[5]; in the subsequent 20 years, methods have been developed for manipulating these polymers to optimize the release kinetics, biocompatibility and degradability[6]. This article will focus on development of polymer-encapsulated agents that elicit longer-lasting, enhanced passive or active immunity.

Passive immunization

Passive immunization is the administration of antibodies to a host in order to neutralize a pathogen; this can be used to protect against acquiring a disease, as postexposure prophylaxis or sometimes as a treatment. The concept of passive immunization was first demonstrated in the late 1800s, when the transfer of serum from an immunized to a nonimmunized individual was shown to confer immunity[7]. Injections of serum gamma globulin are currently used for the prevention of hepatitis A and B infections, the prevention and treatment of measles, and as therapy for chronic immunodeficiency syndromes[8]. Protection of monkeys against HIV-2 or SIV has been demonstrated after systemic intravenous administration of antibodies[9]. Although monoclonal antibody (mAb) technology permits the production of highly specific antibody preparations, their application to disease prevention may be limited by the duration of protection, which lasts only as long as the antibody remains in the body, usually days to weeks.

Systemic delivery

The lifetime of a single dose of antibody can be increased by incorporating the antibody into implantable or injectable polymeric formulations (Fig. 1). For example, antibody was released for over two years after incorporation into a poly(ethylene-co-vinyl acetate) (EVAc) matrix[10]. Subsequently, other delivery systems, including systems composed of biodegradable polymers, have been used for the

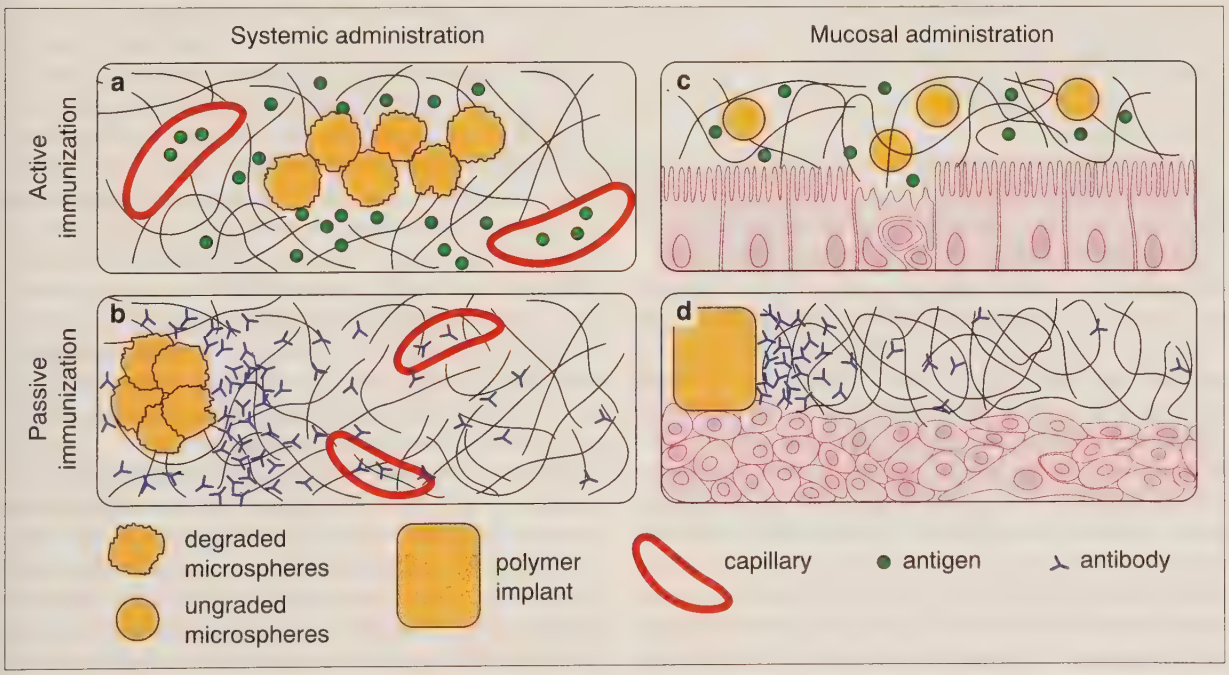

Figure 1

Cartoons depicting the use of polymer delivery systems for the systemic (**a**,**b**) and mucosal (**c**,**d**) delivery of agents for active and passive immunization. Active systemic immunization (**a**) can be achieved after subcutaneous injection of microspheres, either degradable or non-degradable, that release antigen to diffuse into the blood stream, while mucosal stimulation with antigen (**c**) can be achieved when the orally administered microspheres reach the Peyer's patches. Passive delivery of antibodies can be attained by injection (**b**) of microspheres that release antibody molecules, which then migrate into the circulatory system, or insertion of a polymer (**d**) into the female reproductive tract that releases neutralizing antibody into the mucus.

Table 1. Immunization schedules for some common vaccinations[22]

Vaccine	Recommended immunization schedule	
	Primary [Doses:time interval (yr)]	Booster
Diphtheria, tetanus, pertussis (DTP)	4:1	1 dose between ages 4 and 6; every 10 years following with Td[a]
Live poliovirus	4:1.5	4–6 years of age
Killed poliovirus	4:1.5	4–6 years of age and every 5 years to age 18
Measles, mumps, rubella (MMR)	1:n/a	[b]
Hepatitis B	3:0.5	unknown

[a] Td, tetanus and diphtheria toxoids for adult use.
[b] Revaccination necessary if vaccinated prior to 12 months of age.

prolonged release of controlled quantities of active antibody[11]. Antibodies can be incorporated into polymeric microspheres that can be suspended and injected as a single dose (Fig. 1a). Microspheres, usually ranging from 100–200 μm in diameter, can be produced from a variety of polymeric materials – polymers of lactic and glycolic acids are the most commonly used. The kinetics of protein release from the microspheres can be altered by modifying the polymer preparation[12], by varying the relative percentages of the copolymer[13] or by changing the size of the microspheres[14] (Fig. 2). When antibody-loaded poly(L-lactic acid) (PLA) microspheres were injected subcutaneously in a rat, antibody concentrations in the serum were observed for an extended period (Fig. 3). This ability to control the systemic lifetime of an antibody could provide sustained passive immunity.

Mucosal delivery

The vaginal mucosa is an interesting site for the study of antibody delivery (Fig. 1d). Antibodies can be used to neutralize pathogens for STD prevention[16] or to interfere with sperm–egg interactions for contraception[17,18]. When antibody-loaded EVAc vaginal rings were inserted in female mice, high levels of antibody were maintained in the vaginal fluids for one month[19]. In comparison, topically administered aqueous antibody solutions last for only 24 h[20]. The rate of antibody release from the vaginal ring can be controlled by changing the properties of the polymer or the polymer–antibody formulation[11]. These polymeric devices, formed into vaginal rings, have been used to provide long-term passive immunity against genital herpes infections in mice by incorporating anti-herpes virus mAb[21]. Vaginal rings provided significant protection against intravaginal challenges with herpes virus, even seven days after insertion.

Active immunization

The ability of the immune system to respond to antigen is essential for vaccine technology. This response is variable and the immune system usually requires more than one exposure to antigen to provide full immunity to a live pathogen (Table 1). The repeated vaccinations needed to ensure complete immunity in children or adults is not feasible in many parts of the world. Therefore, in an attempt to reduce the inconvenience and expense of repeat immunizations, research has been focused on optimizing the exposure to antigen after a single dose.

Systemic immunization

The first studies of immunity after implantation of an antigen-releasing polymer were published almost 20 years ago[23]. An EVAc polymer pellet less than 1 mm in diameter was implanted subcutaneously in a mouse; prolonged release of antigen produced an antibody titre similar in magnitude and duration to a conventional vaccination with adjuvant. Because EVAc is nonbiodegradable, the pellet would need to be removed after exhaustion of antigen, and so biodegradable polymeric devices, which would dissolve in the body after antigen release, may be more desirable in certain situations. If the polymer-degradation products act as an adjuvant, the polymer could potentially provide two immunity-enhancing elements. When bovine serum albumin (BSA) was incorporated into poly[(N-benzyloxycarbonyl-L-tyrosyl-L-tyrosine hexyl ester)-iminocarbonate] pellets and implanted subcutaneously in mice, significantly higher antibody titres were observed than with a control biodegradable polymer with no adjuvant effects[24].

Antigens can also be incorporated into poly(D,L-lactide-co-glycolide) (PLGA) or PLA microspheres for controlled release. Changing the antigen-release rate, which can be accomplished by the methods described above (Fig. 2b), can alter the subsequent immune response. For example, a peptide that induces a strong T-helper-cell response was incorporated into PLGA and subcutaneously injected into a mouse[13]. Three different formulations of PLGA were tested, and the polymer with the fastest release kinetics produced the highest titre; this immune response was comparable to the response after inoculation of peptide in adjuvant. In another study, the molecular weight and composition of PLA or PLGA (each at a M_R of 3000 or

100 000) was found to alter the release of tetanus toxoid *in vitro*[25]. Following immunization, both formulations of PLGA-encapsulated toxoid produced IgG antibody titres similar to alum-adsorbed toxoid and persisted for at least 26 weeks, while the PLA-encapsulated preparations had significantly lower antibody titres after eight weeks.

Although ovalbumin (OVA) is a poor immunogen when injected alone, a significant immune response can be obtained by the incorporation of OVA into injectable PLGA[26] or PLA[27] microspheres. The increased serum-antibody titres to this antigen appear to be independent of microsphere size, polymer composition or route of administration[26,27]. Delivery of PLGA-encapsulated staphylococcal enterotoxin B (SEB) also produced higher and longer-lasting antibody titres than soluble toxoid[14].The encapsulated antigen appears to have an adjuvant-like response, with IgG titres comparable or higher than those following immunization with antigen in Freund's complete adjuvant[14,26].

Polymeric delivery systems can also enhance the immune response to recombinant peptide antigens: PLGA systems containing peptides carrying both T- and B-cell epitopes produced an immune response comparable to or higher than peptide-adjuvant combinations[13]. The release of recombinant MN gp120 (a protective vaccine for HIV-1) from PLA produced a response similar to the gp120–adjuvant immunization-booster schedule, with neutralizing antibody titres increasing for more than a year[28]. The increased response occurs because the bulk erosion of PLA allows for pulsatile release of antigen (Fig. 3b), so that a single injectable formulation can provide multiple phases of antigen release. A single-shot systemic immunization by polymeric delivery that provides multiple 'bursts' of antigen release is a possibility for antigens that would otherwise require multiple dose vaccinations (Table 1).

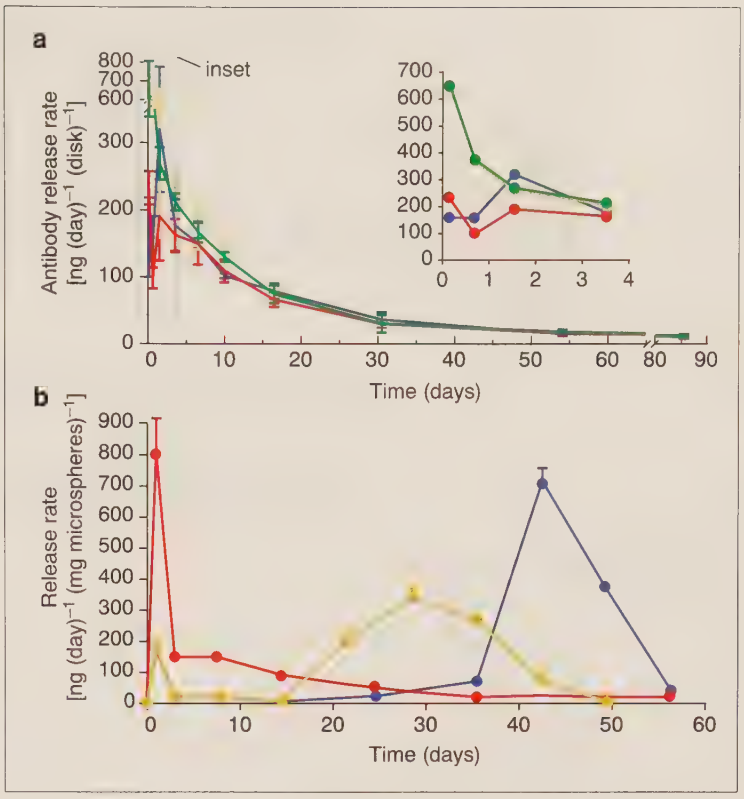

Figure 2

Vaccine-release rates from polymeric devices into buffered saline. (**a**) Varying molecular weight of the dispersed agent alters the initial rate of release. IgG (blue), IgM (green), $F(ab)_2$ (red) and Fab (yellow) release from an EVAc polymer disk codispersed with 400 kD Ficoll (Sherwood, J., 1996, PhD Thesis, The Johns Hopkins University, MD, USA). (**b**) Altering the polymeric composition and molecular weight changes the release rate of antigen. Release rates of a malaria antigen from PLGA (14 kD) (yellow), PLGA (17 kD) (blue), and PLA (130 kD) (red) microspheres. The differences in release peaks can be used to develop vaccines with self-boosting capabilities. Error bars indicate standard deviation. Reproduced from Ref. 15 with permission.

Mucosal immunization

Mucosal immunity, particularly the accumulation of pathogen-specific secretory-IgA (SIgA) antibodies in the mucosal fluids, is essential for protection against many pathogens, but mucosal immunity is difficult to stimulate by conventional methods of antigen administration. Typically, the mucosal immune system needs specific activation to produce protection at a local site, such as the bronchial, reproductive or intestinal tract. Oral administration of microsphere-encapsulated antigen has been shown to produce both a systemic and a mucosal response[29]. Microspheres smaller than 5 μm in diameter appear to be transported through the lymphatic system within macrophages, while those between 5 and 10 μm in diameter remain fixed in the Peyer's patches[29] (Fig. 1b). These findings illustrate the dual potential of oral immunization of vaccine-loaded polymer microspheres – both mucosal and systemic activation can be achieved by controlling the physical characteristics of the microsphere preparation.

Comparison of oral immunizations of soluble and microencapsulated antigen demonstrates the efficacy of microencapsulation. Following oral administration, anti-SEB titres of IgM, IgG and IgA increased after each of three immunizations with PLGA-encapsulated toxoid, while soluble toxoid produced only an IgM-type anti-SEB response[30]. The levels of SIgA were also greatly enhanced in both the saliva and gut washings following tertiary immunization with encapsulated SEB[30]. Oral administration of OVA incorporated in PLGA produced effects comparable to the systemic administration of microspheres (see above), but also produced significant levels of SIgA in the saliva and has been demonstrated to induce a cytotoxic lymphocyte response[31]. Orally-delivered encapsulated OVA was also found to activate a Th2-cell-dependent immune response in the spleen[32] and a Th2- and B-cell immune response in the peripheral and mucosal lymphoid tissues[33].

The concept of both primary and follow-up immunizations with microspheres has been investigated using formalin-treated simian immunodeficiency virus (SIV) as the antigen[34]. Initial injected immunizations, followed by mucosal boosting (which was achieved by either oral or intratracheal administration of

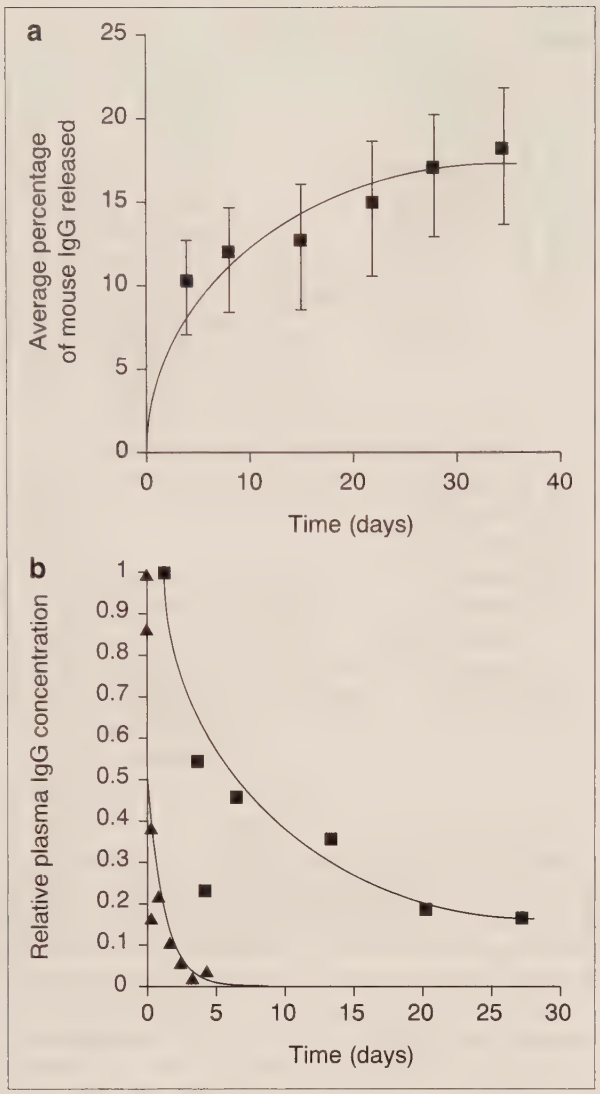

Figure 3

Release of mouse IgG antibody from PLA polymers. (**a**) Average percentage of IgG released from PLA microspheres *in vitro* into phosphate-buffered saline. Error bars illustrate standard deviation. (**b**) Relative plasma IgG concentrations *in vivo* after release from subcutaneously implanted PLA microspheres (■). The half-life of intravenously administered IgG (▲) is less than 1 day in a rat (Dodrill, A. and Saltzman, W. M., unpublished).

microspheres), provided protection from vaginal challenge with SIV, confirming the observation that stimulation of one mucosal site can provide immunity at other mucosal sites. In another example, oral administration of multiple doses of a PLA-encapsulated anti-idiotype vaccine provided protective immunity against ocular challenge with *Chlamydia trachomatis*[35].

Future direction and conclusions

In spite of tremendous medical advances in this century, infectious diseases remain the most important worldwide public-health problem. Immunization represents our best opportunity to control the spread of infectious disease, but we must find methods for making vaccines that provide protective immunity while remaining low in cost and easy to distribute. For example, a major obstacle to the worldwide control of both hepatitis A and hepatitis B is cost, although there are effective vaccines for both[36]. Polymeric delivery systems potentially allow for low costs as a result of their ease of both administration and distribution, as polymer-encapsulated vaccines can be self-administered orally and have extended shelf lives. Cost-effectiveness also requires new methods for the large-scale production of vaccine proteins. Plants may prove to be inexpensive sources of both recombinant antibodies[37] and antigens[38]. Antibodies produced in transgenic plants ('plantibodies') are being tested clinically for prophylaxis against oral pathogens[39]. In addition, hepatitis B surface antigen and bacterial antigens have been produced in transgenic tobacco plants; these proteins appear to be as immunogenic as recombinant proteins produced in yeast or bacteria[38,40]. We believe that the combination of low-cost production of immune-related proteins with appropriate polymeric delivery technology may lead to new opportunities in the control of infectious diseases.

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Developments and applications of biosensors in food analysis

John H. T. Luong, Pierre Bouvrette and Keith B. Male

The food industry needs suitable analytical methods for process and quality control; that is, methods that are rapid, reliable, specific and cost-effective in their provision of information about physical and chemical characteristics of food. Apart from a few important analytes, such as sugars, alcohols, amino acids, flavours and sweeteners, food applications mainly focus on the determination of contaminants. However, very few biosensors play a prominent role in food processing or quality control. Considerable effort must be made to develop biosensors that are inexpensive, reliable, and robust enough to operate under realistic conditions.

Reliable and cost-effective analytical methods are increasingly needed in the food industry for the determination of specific chemical compounds in foods and food products. The need arises from increased regulatory action and heightened consumer concern about food composition and safety. It has been estimated that the food industry spends, on average, 1.5 to 2% of the value of its total sales on quality control and appraisal. For example, several million penicillin assays for mastitis are performed on cattle milk in US dairy farms every year. Contamination of foods by toxins (e.g. domoic acid in mussels, tetrodotoxin in fish), viruses (e.g. hepatitis A) or microorganisms (e.g. *Escherichia coli*, *Salmonella*, *Listeria*) is a serious concern. The recent bovine spongiform encephalopathy (BSE) outbreak in the UK, as a result of which several million cattle and dairy products were ordered to be destroyed, has not been quickly forgotten. According to the Center for Disease Control and Prevention (Atlanta, USA), 6.5 million illnesses and 9000 deaths occur every year in the USA because of foodborne bacterial contamination[1]. Food products now require extensive labelling of all their major and minor constituents – artificial flavours and sweeteners, antimicrobial agents and allergens, glucose in sugars and syrups, alcohols in wine, hydrogen peroxide and sulphites as preservatives, antibiotics in milk, cholesterol, glutamate and vitamins, to name just a few. The contribution of analytical services in the food industry is also becoming increasingly important in the monitoring of raw material conversion and composition, verification of product contents and evaluation of product freshness (see Table 1 for more applications). Various analytical methods have been developed and applied to food analysis that can provide information concerning the exact physical and chemical characteristics of food, including data about contaminants. However, conventional methods such as chromatography are time-consuming, requiring sample pretreatment, expensive equipment (e.g. multidetector systems, chromatographic columns) and high-purity chemicals as mobile-phase modifiers.

Biosensors, an amalgamation of signal transducers and biocomponents, are expected to play a prominent role in food processing and quality control. Compactness, portability and high specificity and sensitivity are among the reasons that biosensors provide considerable promise and potential for replacing current 'wet'

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PII: S0167-7799(97)01071-8

Table 1. Areas of application for biosensors

Target compounds	Areas of application and comments
Organic	
Amino acids, cholesterol, carbohydrates, pesticides, antibiotics, alcohols, vitamins, carboxylic acids, phenols, lipids, lecithin	Common constituents or contaminants in food products.
Lysine	Marker of nutritional damage due to heat.
L-alanine (with D-alanine)	Flavour enhancer.
Citrate	Found in several fruits and in all animal and vegetable cells.
Catechins, catechols and tannin	Taste and function of green tea. Quality control in tea processing (substances of astringency).
Polyphenols	Olive oils (taste and stability of the oil).
Acetaldehyde	Wine, beer, yoghurts.
Malolactic acid	Wine quality.
Trimethylamine, putrescine, cadaverine and histamine	Meat spoilage and aging, histamine in red wine.
Nucleotides: hypoxanthine, inosine, inosine-5-monophosphate	Fish freshness, meat aging.
Salicylate	Antimicrobial agent, preservative (now forbidden in most countries).
Benzoic acid, sorbic acid, tocopherol	Preservatives.
Glutamate	Flavour enhancer.
Lactate	Yoghurt, beer, fruit juices, wine.
Amygdalin	Cyanide-containing sugar in almonds.
Artificial sweeteners (aspartame, saccharin, cyclamate, acesulfame, etc.)	Soft drinks, desserts.
Glucosinolates	Nitrile-and sulphur-containing heteroglycosides.
Inorganic	
Sulphites, sulphur dioxide	Used as food preservatives, oxidation prevention.
Potassium, sodium, calcium, magnesium, nitrate, nitrite, chloride, sulphate, fluoride, carbonate, zinc, mercury	Vinegar, fruit juices, milk, soft drinks, mineral water.
Toxins and foodborne pathogens	
Saxitoxin, neosaxitoxin, gonyautoxins, domoic acid, brevetoxin, Protein A, hepatitis A virus, aflatoxin, ciguatoxin	Protein A is a product of *Staphylococcus aureus*.
Tetrodotoxin	Marine shellfish poisoning.
Salmonella, *Escherichia coli*, *Listeria*, *Campylobacter*, *Staphylococcus*, *Yersinia*, bacteriophages	In a variety of foodstuffs.

chemistries and analytical practices. Since the pioneering work of Clark and Lyons in 1962 (Ref. 2), there has been a phenomenal growth in the field of biosensors. However, although considerable research on biosensors has focused on concept demonstration, little or no attention has been paid to the harsh conditions under which the biosensors were supposed to operate. In many cases, the biosensor was never challenged with real samples, and the gap between academic biosensor developments and practical applications remains wide. Another disappointing fact is that very few biosensor-based products have been commercialized and none are exclusively dedicated to the food industry. Biosensor development has generally focused on clinical and other non-food areas, although the analytes studied may also be found in foods. Most biosensors for food applications that have been reported in the literature have been developed for the determination of carbohydrates, mainly glucose. Many promising biosensors fail miserably during their first contact with food samples, due largely to the high complexity of the samples involved. This article will address the development of biosensors and discuss the technical and economic problems of applying this technology to the monitoring of food-related products.

General aspects of biological materials and immobilization technology

There are at present over 2500 known enzymes, with oxidases the most favoured biocatalyst[3], owing to the fact that they exhibit high stability, thus having a long lifetime, and require no coenzymes or cofactors, as is the case with dehydrogenases. A critical factor for optimal biosensor design is maximal retention of the bioactivity and biostability of the biological molecule, confined in a thin layer as close as possible to the transducer (Fig. 1). A crucial part of this is effective enzyme immobilization that still allows free diffusion of substrates and products into and out of the enzyme layer. The concept of using immobile biological molecules is appealing because this permits the reuse of the molecules and allows significant simplification and automation of the analytical apparatus, all contributing to reduced costs.

Numerous methods have been developed for biomolecule immobilization – for example, adsorption, entrapment in a gel lattice, covalent binding to activated groups on the support and the use of a cross-linking reagent[4]. In general, biosensors using adsorbed enzymes or other proteins usually give poor performance, and, with the exception of a few cases, this procedure alone is rarely used in biosensor construction. Intramolecular enzyme cross-linking by a multifunctional reagent is also not effective unless it is used in conjunction with covalent binding or entrapment procedures.

Many biosensors entrap an enzyme within a semipermeable membrane to minimize the steric, diffusion and denaturation problems that occur with chemical methods. The choice of semipermeable membrane may have a significant effect on the sensitivity and background of the resulting signal. A good example is the use of Nafion™ (a highly negatively charged, perfluorinated sulfonate polymer) to suppress interference by ascorbic acid, uric acid and acetaminophen, which, due to the charge repulsion between these compounds and the membrane, are hindered from reaching the electrode and causing interference[5].

One of the most commonly used methods is the electrochemical polymerization of organic molecules to form a polymer network (either conducting or nonconducting), entrapping the enzyme[6]. This technique allows microfabrication, mass production and more precise control of film thickness, homogeneity and reproducibility. It has been shown that electropolymerized nonconducting films possess unmatched interference-rejection characteristics for ascorbate, urate, cysteine and paracetamol, and, being very thin, give rise to fast-response (i.e. within a few seconds) and highly sensitive sensors[7–9]. A similar approach involves the deposition of a thin metal film over a layer of enzyme molecules adsorbed to an appropriate base support. Functioning biomolecules can also be encapsulated in silicate glasses using the sol–gel method[10]; these matrices are transparent, permitting optical monitoring of the spectroscopic properties of the entrapped molecules for the transduction of chemical interactions. However, this technique has the major disadvantage that the structure and properties of the gel continue to change as long as the solvent remains in its pores.

Other sensors are based on covalently coupling a biocomponent to a porous membrane attached to the sensing area of an electrode. Functional groups available in enzymes mainly originate from the amino acid side chains: ϵ-amino groups from lysine, carboxyl groups from aspartate and glutamate, sulfhydryl groups from cysteine, phenolic hydroxyl groups from tyrosine. The commercially available preactivated membranes [Immunodyne™, (Pall BioSupport, NY, USA) and Immobilon™ (Millipore, MA, USA)] are suitable for the preparation of any bioactive membrane, because direct contact between the enzyme and the membrane is all that is required for binding. The enzyme can be cross linked to a protein such as bovine serum albumin to form a thin layer on the support surface to enhance the enzyme loading[11]. Numerous bifunctional reagents are available for this purpose, including glutaraldehyde, the most common cross-linking agent, which often reacts with the ϵ-amino groups of lysine in an enzyme.

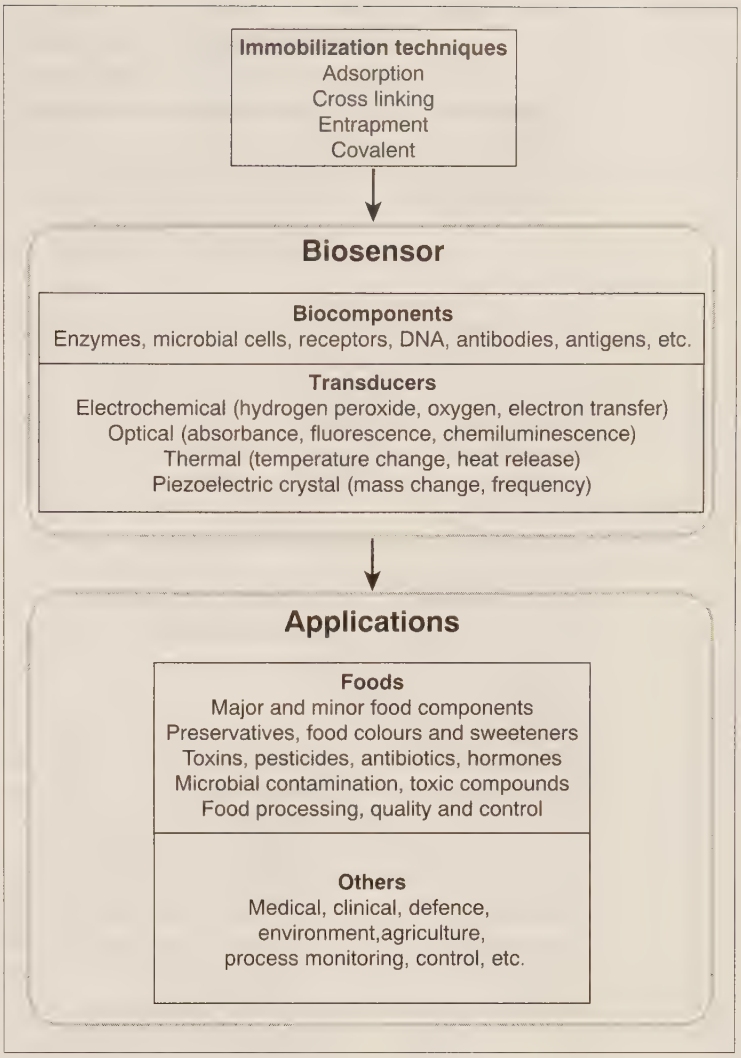

Figure 1
Biocomponents and transducers commonly used in biosensor construction. A biological element (e.g. enzyme, microbial cell, antibody, receptor) is coupled to a transducer to convert a selective biochemical response into a quantifiable electrical signal.

In the flow-injection-analysis system or the enzyme thermistor, the enzyme reactor is often inserted into a flow-through system and the reaction products then measured by an appropriate downstream detector. Typical support materials are: activated controlled-pore glass; activated Sepharose; ion-exchange resins; and oxiran-acrylic beads. The enzyme-reactor system is especially useful in cases where the enzyme has low specific activity and/or high Michaelis–Menten constant (K_M). Enzymes can also be covalently immobilized onto water-soluble particles and held between two selectively permeable membranes. A number of such proprietary combination membranes have been developed and commercialized. The Yellow Springs Instruments (OH, USA) glucose and lactate membranes

Table 2. Advantages and disadvantages of different biosensor systems

Transducer	Advantages/disadvantages [(+)/(–)]	Applications
Ion-sensitive electrode (ISE)	(+) simple, reliable. (–) sluggish response, requirement for a stable reference electrode, susceptible to electronic noise.	Glutamate, urea, nitrite, amino acids. Penicillin, DNA, RNA and glucose (pH-based ISE). Glucose, amino acids, cholesterol and alcohols (iodide-based ISE). Amygdalin (cyanide-sensitive ISE).
O_2 electrode	(+) simple, high selectivity. (–) low sensitivity.	Mainly developed for glucose, sulphite, glutamate, ethanol, oxalate, L-amino acids, tyrosine, monoamine (together with an oxidase or a microbial cell), sucrose, lactate, cholesterol, aspartame, nucleotides, galactose, acetic acid, lysine, biological oxygen demand, etc.
H_2O_2 electrode	(+) simple, high sensitivity. (–) low selectivity.	Sulphite, glucose, cholesterol, glutamate, nucleotides, putrescine, cadaverine, histamine, L-amino acids, aspartame, lactate, glutamine, alcohols, etc.
Optical systems	(+) remote sensing, low cost, can be miniaturized, free from electrical interference. (–)require high-energy sources, only applicable to a narrow concentration range, interference from ambient light.	Acetaldehyde, alanine, malate, lactate, nitrate, glucose, glycerol, ethanol, xylitol, isocitrate, glutamate, sorbitol, galactose.
Piezoelectric	(+) fast response, simple, stable output, low cost for readout device, no special sample handling. (–) low sensitivity in liquid applications, interference due to nonspecific binding.	Glucose, atrazine, propazine, signal, simazine, *Salmonella*, *Escherichia coli*, *Candida*, other microorganisms and microbial toxins.
Calorimetric	(+) versatility, free from optical interferences such as colour and turbidity. (–) expensive, cumbersome, require a large amount of enzyme.	Several compounds, such as ascorbic acid, glucose, lactate, triglycerides, cholesterol, galactose, ethanol, sucrose, penicillin G, oxalate, lipids, xanthine and hypoxanthine.
ISFET (ion-sensitive field-effect transistor)	(+) low cost, mass production, stable output, requires very small amount of biological material, monitors several analytes simultaneously. (–) temperature sensitive, fabrication of different layers on the gate has not been perfected.	Glucose, lactate, urea and several ions.

have been prepared in this way, using a cellulose-acetate–polycarbonate combination with the enzymes covalently immobilized on glutaraldehyde-activated resin particles.

Transducers used in biosensor construction

The activity of a biological component towards its substrate can be monitored by oxygen consumption or hydrogen-peroxide production, NADH fluorescence or absorption, or changes in pH, conductivity, temperature or mass. Several types of transducer have been used in biosensor construction (Table 2).

Electrochemical transducers

Amperometric (e.g. oxygen or hydrogen peroxide) and potentiometric (e.g. pH and carbon dioxide) transduction have found the widest applications. The combination of oxidoreductases and amperometric electrodes gives the best results, because the enzymatic reactions with their substrates may be measured both easily and sensitively. This combination constitutes one of the most successful classes of biosensors, with sensors available for over 80 substrates[12]. Of these compounds, several could be of interest for the food industry (Table 2).

The main advantage of hydrogen-peroxide-detection over oxygen is higher sensitivity, but the oxygen electrode is the only option available when microbial cells or plant or animal tissues are used as the main biological components. Antibodies labelled with oxidases have also been used mainly in the determination of antigens, and the sensors are known as 'amperometric enzyme-linked immunosensors'.

Optical transducers

Optical sensors, originally developed for oxygen, carbon dioxide and pH, have been extended to the construction of fluorescent and luminescent optrodes. The selective biocomponent is immobilized at one end of an optical fibre, with both the excitation and detection components located at the other end. $NAD(P)^+$/NAD(P)H-dependent dehydrogenases are obvious candidates for these biosensors, because NAD(P)H is known to absorb light strongly at 340 nm and fluoresce at 460 nm. Several $NAD(P)^+$/NAD(P)H-dependent dehydrogenase enzymes exist for a number of food- and beverage-related compounds (Table 2), but the requirement for expensive and unstable cofactors (NAD^+/NADH or $NADP^+$/NADPH) is the major drawback of this technique. Laser fibre optics allows high intensity light to travel a long distance using a fibrous carrier. Hydrogen peroxide fibre-optic sensors based on absorbance or chemiluminescence have also been developed for various analytes using oxidases. Optical transducers can also take advantage of affinity interactions between antibodies and antigens to screen for microbial contaminants in food.

Thermal transducers

This device is regarded as a small microcalorimeter with immobilized biocomponents, packed in a small column in proximity to the heat-sensing transducer, commonly a thermistor. Most enzyme- or microorganism-catalysed reactions are accompanied by considerable heat evolution (20–100 kJ mol^{-1}), and so this device has a broader applicability than other transducers[13]. The commercial enzyme thermistor (ThermoMetric Inc., Jarfalla, Sweden) claims to detect temperature changes in the range 0.0001–0.05°C. The sensitivity of this technique is equivalent to a substrate concentration of about 10^{-5} M, which is considered satisfactory for the analysis of several major food components. Thermal transducers have also been applied to the detection of antibody–antigen interactions, and this technique is known as the 'thermometric enzyme-linked immunosorbent assay'. However, the use of sophisticated and expensive instrumentation is the major drawback of this technique.

Piezoelectric transducer (quartz microbalance)

A quartz crystal can oscillate when partially or completely immersed in a liquid, and if the physicochemical properties of the liquid (such as viscosity, density and conductivity) are defined, a change in the weight of the quartz crystal can be detected by measuring its vibration frequency[14]. Biologically active materials such as antibodies, enzymes and antigens have been used as active coatings. For the past two decades, intensive research has been directed to developing piezoelectric sensors for a variety of applications, including food analysis[15] (Table 2). However, nonspecific binding and poor sensitivity are the two major drawbacks of piezoelectric transduction.

Potential applications and commercially available biosensors for the food industry

The food industry is very large, but its profit margins are very low and competition is fierce. Consequently, this industry cannot afford to invest in modern, expensive analytical methods at the same level as other high-technology sectors. The industry produces a multitude of different products, and there are limited openings for a major new analytical device. New legislation and regulation may open up some markets for biosensors in this industry, but biosensors will still have to compete with other analytical methods in terms of cost, performance and reliability. Gas chromatography, high-performance liquid chromatography (HPLC) and capillary electrophoresis have been getting better, smaller and more affordable. These analytical systems are able to detect several compounds simultaneously and thus improve the reliability and speed of analysis.

The most promising opening in the food industry is perhaps the rapid detection of microbial contamination and toxins, but it remains to be seen whether a biosensor can compete with other technologies, including the automated immunoassay, for this particular application. Biosensors for in-line (the sensor is brought to the sample) or at least on-line (the sample is brought to the sensor) analysis may also find several applications in the food industry to facilitate the quality control of products during processing. In process control, biosensors offer two distinct advantages – high selectivity and rapid response in comparison with chromatographic methods. To date, biosensors cannot be sterilized and only function within a limited concentration range. In addition, recalibration and/or replacement of their biocomponent must be done frequently and sensor fouling still represents the main drawback for using biosensors *in situ*. However, microdialysis (a technique based on the passive diffusion of analytes across a semipermeable membrane) has been coupled with biosensors as a possible alternative to the use of biosensors *in situ*. Combining microdialysis sampling with flow-injection analysis has been used for the continuous monitoring of glucose and lactate in complex media such as serum, milk and yoghurt[16,17].

Very few biosensors are presently used in the food industry for off-line analysis, although in principle they can be combined with flow-injection analysis for on-line monitoring of raw materials, product quality and possibly the manufacturing process[18]. In general, commercially available biosensors for applications in the food and beverage industry are based on very similar technology, either an oxygen electrode or a hydrogen-peroxide electrode in conjunction with an immobilized

Table 3. A selection of companies that have been involved in the development and commercialization of biosensors for use in the food industry

Manufacturers/Instruments	Target compounds	Comments
Yellow Springs Instruments (YSI) Analysers (Yellow Springs, OH, USA)	Glucose, lactate, ethanol, sucrose, lactose, glutamate.	The analytes are quantified within 1 min of injection. Hydrogen peroxide is detected electrochemically. Some two-channel models are available.
Glucoprocesseur (Solea-Tacussel, Villeurbanne, France)	Glucose and lactate.	The detection principle is similar to the YSI analysers. Glucose and L-lactate can be assayed every 90 sec.
EKSAN Enzyme Analysers (patented in former USSR)	Glucose, lactose, sucrose, L-lactate, ethanol and amino acids.	Hydrogen peroxide is detected. The sampling frequency is up to 40 hr^{-1}.
ESAT Glucose Analyser (Eppendorf, Hamburg, Germany)	Glucose.	To detect hydrogen peroxide. Each glucose-oxidase membrane is reusable for over 2000 assays.
Apec Glucose Analyser (Danvers, MA, USA)	Glucose.	Oxygen consumption is detected.
Biometra Biosensors for HPLC (Biometra Biomedizinische Analytik GmbH, Göttingen, Germany)	Glucose, methanol and ethanol.	Separation by HPLC with postcolumn biosensor detection.
Toyo Jozo Biosensors (Tokyo, Japan)	Glucose, lactate, cholesterol, triglycerides and phospholipids.	Enzymes are immobilized in front of an oxygen electrode.
Oriental Freshness Meter (Niiza Saitama, Japan)	Fish freshness (adenosine triphosphate degradation).	Using soluble enzymes and an oxygen electrode.

oxidase. They are available in several forms, such as autoanalysers, manual laboratory instruments and portable (hand-held) devices. In addition to glucose, lactate, alcohols and amino acids, biosensors have been developed for sensing aspartame, glutamate, lipids and cholesterol. Such biosensors only detect one or two target analytes without having to change the biocomponent, and they cannot be easily integrated with other analytical systems to perform multiple analyses. As an added difficulty, ascorbic acid and vanillin have frequently been encountered as major interferents. So far, simple dipstick tests are easier, more user friendly and less expensive than portable biosensors for field testing, whereas automated-immunoassay and chromatographic methods are still the techniques of choice for multiple samples and analyses. This is the main reason why there is only limited acceptance of biosensors in the food industry. A description of the activities of selected companies that have been involved in the development and commercialization of biosensors for use in the food industry is shown in Table 3.

Development trends and prospects

Prospects for biodetection elements

Amperometric electrodes using oxidase systems will continue to have a dominant position in commercial biosensors, but the search will continue for new oxidases[19]. Dehydrogenases are used less extensively and this trend will not be reversed unless an efficient method can be developed for the regeneration of their cofactors. Microbial biosensors and tissue-based biosensors deserve a brief mention here, because their use removes the requirement to obtain purified biomaterial. The main drawbacks of such biosensors are their poor selectivity and slow response. This type of biosensor can only be used for well-defined samples and will probably remain in the laboratory as a useful research tool. Chemoreceptors react very specifically with their substrates, but their use in biosensor construction is still extremely limited. Their function has not been extensively investigated and the isolation and purification of such fragile biomolecules is still a formidable task. Nevertheless, bundles of nerve fibres isolated from *Calinectes sapidus* (the blue crab) antennule olfactory chemoreceptors have been reported to respond extremely rapidly to amino acids[20].

Increasing the purity of enzymes would lead to the possibility of decreased analysis times, because a higher loading of the active enzyme would be obtained per unit area of membrane surface. This can be seen from a recent report on the purification of a diamine oxidase for the measurement of cadaverine and

putrescine[21]. Improvement in immobilization technologies should also result in increased enzyme loading, for example by increasing the number of binding sites on the surface using avidin and then biotinylated enzymes.

To make biosensors more affordable, efforts must be focused on improving the long-term operational and storage stability of the biosensors. Ideally, the shelf life of the biocomponent must be several months, if not years, but, with the exception of glucose oxidase and few other oxidases, most of the enzymes are not very stable. It has been anticipated that this problem can be overcome by using enzymes that are stable at high temperatures, naturally available from thermophilic microorganisms, but such enzymes have not been used in biosensor construction. Chemical modification of enzymes to make them more stable and more specific is another interesting approach. One of the best examples of this is the use of deflavinated xanthine oxidase to overcome oxygen interference in the construction of a mediated amperometric biosensor for xanthine and hypoxanthine[22]. Protein engineering will lead to a better understanding of protein stability by studying the effects of amino acid changes, and thus to the production of more stable biocatalysts. In addition, the modification of a single charge in the vicinity of the active site of an enzyme can have a significant effect on the pH dependence of the catalytic reaction[23]. This approach is particularly useful for biosensor construction using bi- or multienzymes with different pH optima.

For most analytical purposes, sufficient enzyme can be immobilized that only a relatively small proportion needs to be active for the sensor to be fully functional. In some cases, however, a large fraction must be active. If this is the case, it may be useful to immobilize the enzyme with the active site facing away from the electrode surface. With large substrates, this may be particularly true, because the matrix surface may physically block access of the substrate to the active sites that are even partially obstructed by poor orientation on the surface. As an example, antibodies immobilized by their Fc region have twice the binding capacity of randomly immobilized antibodies[24]. Combining protein engineering and new coupling techniques capable of immobilizing specific sites on the protein to the support surface may lead to new and more effective biosensors.

While still in their infancy, some current studies have focused on the production of taste and smell receptors or receptor arrays, because biosensors of this type could have a number of applications in the food industry. Piezoelectric fragrance biosensors have recently been developed by two Japanese companies (Sogo Pharmaceutical and Toyo) that use a synthetic bilayer film functioning like human olfactory cells for the determination of odour substances. Chemicals such as benzyl acetate, 1-undecanol and *p*-anisaldehyde can be detected within 3 minutes. A multichannel sensor has also been developed to evaluate taste patterns[25]. Lipid membranes were used as sensing elements, with an electrode to recognize five basic tastes including 'umami', a taste sensation that is conveyed by several substances occurring naturally in foods, including glutamate. Taste patterns obtained by the biosensor were clearly distinctive, with high reproducibility.

Improvements in transducer technology

Although useful, amperometric electrodes based on oxygen or hydrogen-peroxide measurement have a limited linear range, because of low oxygen concentration, changes in response due to variations in the ambient oxygen concentration and the deactivation of the enzyme by the hydrogen-peroxide product. Another major disadvantage of hydrogen-peroxide electrodes is the magnitude of the applied potential necessary for the oxidation of H_2O_2 (0.7 V), as many other compounds are also oxidized at this level. Permselective membranes, which block the passage of such compounds, can be incorporated into the biosensor, as in the case of the Yellow Springs Instruments glucose analyser. However, this technique will lead to poorer sensitivity because of diffusion problems, and it cannot be adapted for mass production or microfabrication. Another alternative is to replace oxygen in the oxidase reaction with electroactive mediators that can be detected at much lower applied potentials, where the interfering compounds are not electroactive. Electrons would be passed from the reduced form of the enzyme to a small molecular electron mediator such as ferrocene[26], tetrathiafulvalene[27], ferricyanide[28] or benzoquinone[28], which would then in turn be oxidized at a graphite anode. The difficulty is in providing mediators that are sufficiently mobile to allow for the transfer of electrons but will also allow for the stable operation of the sensor. Interference by ascorbic acid and oxygen is still problematical in such biosensor systems. Mediated implantable miniature glucose electrodes have been tested on animals and have potential for implantation in humans[29]. The exploitation of mediators will culminate in more reliable on-line amperometric biosensors in the near future. The application of such biosensors may eliminate the time-consuming extraction procedure, particularly for solid or semisolid samples.

Piezoelectric crystals coated with antibodies, enzymes or organic materials can be used to detect pesticides, hormones, antibiotics and microorganisms, all of which are of special interest to the food industry. They can also be used in conjunction with flow-injection-analysis systems, which should facilitate automation or continuous on-line monitoring. This raises the exciting possibility of using different crystal assays in series to detect various analytes in complex samples, with an on-line display of the results. However, the change in oscillation frequency on immersing crystals in solutions must be better understood. In general, liquid-phase piezoelectric sensors based on antibody–antigen interactions are not suitable for multiple assays, because the bound analyte cannot be easily removed from the crystal surface[30]. This problem may not be as important if small crystals can be

manufactured at low cost, so that disposable transducers are economically feasible.

Thermal biosensors can be used successfully in turbid and highly chromogenic solutions, where electrical and optical systems are limited. Another promising approach is the development of microcalorimetric biosensors, comprising high-resolution, thin-film temperature sensors. Unfortunately, like piezocrystal biosensors, progress in this area is very slow. To date, a major limitation of this technique has been its high cost and the lack of commercial instrumentation.

The use of semiconductor transducers is well matched with the use of bioaffinity proteins. An electrochemical bioaffinity sensor of interest to the food industry has been produced that uses an interdigitated electrode capacitor and the mammalian receptor for acetylcholine[31]. The sensor can be used for the detection of organophosphate and carbamate pesticides. It retained its biological activity for nine months at room temperature and was reusable for 15 repeated applications. Other semiconductor sensors have been produced using valinomycin for K^+, urease for urea, xanthine oxidase for hypoxanthine (an indicator of fish freshness) and antibodies for microorganisms and specific DNA sequences. In the long term, odour and taste receptors may be used to develop biosensors that mimic human sensory capabilities. Such detectors would be useful for monitoring essential oils and flavourings. For most semiconductor sensors, there are still problems with the attachment of receptors to the semiconductor surface, with the protection of the semiconductor from test solutions and the environment, and with loss of sensing material from the interface membrane.

Fibre-optic biosensors measure, either directly or indirectly, the optical signal originating from the target compound, and could prove to be very sensitive. Because they can be used in harsh environments, fibre-optic biosensors should be of particular interest for on-line processes. Many oxidases can be used in the presence of luminol (a compound that luminesces in the presence of hydrogen peroxide) to produce luminescent optical biosensors. Colorimetric and fluorescent indicator dyes that change absorbance or fluorescence with pH or oxidation state lend themselves easily to applications using optical fibres. However, despite its obvious potential, no instrumentation to perform food analysis via fibre-optic biosensors is presently commercially available.

Sample preparation

For many applications, the sample-preparation time, not the biosensor-response time, is the rate-determining step of the process. Many biosensors are vulnerable to extremes of pH and ionic strength and so the sample must be suitably neutralized or diluted prior to measurement. In the case of solid or hydrophobic foodstuffs, a suitable extraction procedure must be developed. For instance, the measurement of nucleotides or diamines in fish tissues requires a time-consuming procedure for the preparation of fish extract[32]. Improvements in this area are of great importance in order to reduce the overall analysis time. The microwave-hydrolysis technique, which has been successfully applied for the determination of amino acids and furosine in food, is a potential candidate[33]. Supercritical-fluid extraction also provides a rapid and reproducible method for preparing nonpolar (e.g. flavour compounds) to moderately polar (e.g. carbohydrates, amino acids) compounds in solid or semisolid foodstuffs[34]. Moving sample preparation closer to the biosensor and using robotics in sample preparation are other elegant approaches to increasing the speed and reliability of sample preparation and analysis. One example is the incorporation of an autosampler with a biosensor to process the sample (dilute, mix, evaporate, filter, extract, etc.) and analyse the results, all directed by the same computer or microprocessor.

Biosensors for water-insoluble compounds are seldom described because of the additional problems of diffusion of the analyte into the immobilized-enzyme layer. This problem can be circumvented if the sensor is operated in an organic solvent[35] or in cyclodextrins, cyclic oligosaccharides that can form inclusion complexes with various organic compounds by incorporating 'guest' molecules into their hydrophobic cavities[36]. Another possibility, which the industry would prefer, is a biosensor that could penetrate the tissue and provide direct readings. This concept would require the utilization of microelectrodes and microfabrication technology, an area of biosensor research that has only emerged recently.

Future possibilities and conclusions

Currently, the food industry is not very receptive to biosensor technology, although a niche market will probably be developed in the long run. However, a method for the rapid detection of microorganisms or toxins is appealing and will probably offer the best opportunity for biosensors in this industry, but several key issues also need to be resolved before biosensors find widespread applications. For head-to-head competition with HPLC, capillary electrophoresis or other analytical procedures, biosensors must be designed to measure several different analytes in parallel using a single analyser without having to change any component of the biosensor. Using a multichannel biosensor system with flow-injection analysis, such a situation has been realized for ions, glucose, lactate and creatinine in clinical chemistry and should be adaptable for the food industry. The next crucial step appears to be the development of improved methods for the mass production and reliable provision of the currently available biological materials. The biosensors should be constructed in a modular fashion to enable them to be integrated with other analytical systems and to carry out multiple analyses. The combination of HPLC or capillary electrophoresis with selective and sensitive postcolumn biosensor detection systems is one distinct possibility, and has already been exploited by several research groups. This is not a speculative method, but offers the possibility of detection and simultaneous

determination of many compounds in foodstuffs. Additional efforts are also being directed at developing portable, low cost and reagentless biosensors other than amperometric electrodes. Fibre-optic sensors, piezoelectric crystals and ion-sensitive field-effect transistors are potential candidates because they can easily be miniaturized and integrated for the simultaneous determination of different target compounds in food samples.

The stability of the immobilized material can be improved by the isolation of thermophilic enzymes and by the discovery of novel stabilizers. Improved immobilization methods are still needed to enhance the loading, activity and stability of the immobilized materials, particularly in the case of membrane-bound receptor proteins. More applications will be envisioned as the search continues for new enzymes, microbial cells, receptors or other biological materials. However, in the short term, research should concentrate on combining known enzymes and approved or promising transducers for the development of 'real-world' applications. An amino acid electrode for the detection of total amino acids was developed in this way using the enzymes L-glutamate oxidase, L-lysine oxidase and tyrosinase[37]. Biosensors must be inexpensive, reliable and robust enough to operate under realistic, and perhaps hostile, conditions, and possibly form part of a highly integrated analytical system. This aspect is both technically challenging and mandatory for a successful commercialization of biosensors in this conservative and competitive industrial sector.

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Cell-biology cookbook

Cell Biology: Essential Techniques

edited by D. Rickwood and J. R. Harris, J. Wiley & Sons, 1996. UK£16.99 (xii + 173 pages) ISBN 0 471 96315 1

Cell Biology is another volume in the *Essential Techniques* series, a series of pocket-sized, spiral-bound manuals aimed at providing researchers in the life sciences with immediate access to regularly used protocols. This volume has six chapters covering three main topics – microscopy (both light and electron), cell culture and different aspects of cell fractionation [the isolation and analysis of organelles and cell membranes, and the isolation and fractionation of (poly)ribosomes]. Each chapter provides background information, followed by clearly written and mostly up-to-date experimental protocols. Often, the notes following the methods provide additional information that would otherwise only be available through experience (time requirements, stability of solutions, incompatibility of reagents, etc.).

Due to the size of the manual (173 pages), it is quite understandable that not all aspects pertinent to the discussed topics are covered. Compared with the large amount of technical information found in the chapters on cell-fractionation techniques, the chapters on microscopy, in particular light microscopy, appear somewhat scanty, perhaps a reflection of the working field and experience of the editors; the addition of some protocols on fixation and general staining procedures for light microscopy (only the setting up of microscopes for different uses is discussed) would be appropriate. In Chapter 3 (for reasons of space), only mammalian cell culture is covered. Given the widespread use of other cell types (plants, insects, microorganisms, etc.) in the life sciences, some reference to books covering these aspects of cell culture might have been useful. As already mentioned, cell-fractionation techniques are extensively treated in this book, but the isolation of organelles from cultured mammalian cells or yeasts could perhaps have been added to Chapter 4. The protocols provided on commonly used marker enzymes (mainly for mammalian tissues) are adequate; the complexity of the Golgi apparatus, however, calls for more than one marker, and the use of antibodies directed against cell compartments in fractionation studies is not considered.

Overall, however, coverage is comprehensive. Considering the price of this manual, it is good value, and the information, presented in a condensed but easily understandable format, will be helpful to most, and particularly to newcomers to the field.

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I wouldn't start from here

Route Maps in Gene Technology

by Matthew R. Walker with Ralph Rapley, Blackwell Science, 1997. UK£19.95 (334 pages) ISBN 0 632 03792 X

For the undergraduate (or even the graduate), the technology of molecular genetics can be daunting. It's easy enough to pick up the buzz words, such as 'PCR' or 'gene therapy', but often, in the student's mind, there is an incomplete conceptual understanding or an imprecise feel for how the particular technique features in the bigger picture. Turning to textbooks for explanations can often be a frustrating business. A glossary entry is usually insufficient and fuller explanations often have to be gleaned by delving into more than one textbook. Wouldn't it be nice if succinct answers could be found between the covers of a single book?

Recognizing the problem, Matthew Walker has attempted (with help from Ralph Rapley) to remedy the situation by producing a book that focuses on 100 key issues in gene technology. These issues are the basis of individual numbered sections, each two or four pages long, that are intended to be 'a series of overlapping "introductions" '. They follow a standardized, if somewhat complicated, format that is explained at the start of the book. Adjacent to the title, at the top of even-numbered pages, there is a (self explanatory) summary box and below there follows an introduction (again, self explanatory), then the main information that actually describes the subject of that section. Halftone figures are used to illustrate concepts. A 'compass box' provides information that helps link the information within that section to other information or ideas. 'Route prompts' at the top of odd-numbered pages provide the actual direct links to other numbered sections. Access to original articles, reviews and suggested further reading is provided by the 'biblio-boxes' at the bottom of even-numbered pages, leading to three extensive bibliographies at the end of the book. The idea is that you can start from any point and navigate your way around any aspect of gene technology using the boxes and prompts.

This combination of approaches ought to work well, but the end effect is sometimes hampered by poor execution and by outdated information. To illustrate this,

consider Section 44 (on Restriction Fragment Length Polymorphism) (RFLP). I feel that it might have been more useful to describe how RFLPs arise before any discussion of their detection, but that is a matter of personal opinion. Rather more serious is the depiction in the accompanying figure (44.2) of the various ways in which RFLPs arise. The upper portion of the Figure shows a progenitor allele (A) and five others (B–F) derived from it in various, explained, ways. The lower portion of the figure then illustrates how the various restriction fragments would appear on an electrophoresis gel (or a Southern blot). Lane C of the gel ought to show four bands but only shows three, and there are bands drawn at incorrect positions in lanes A, E and F. The gel itself is depicted with a linear size scale to its left, suggesting that the separation of DNA fragments by electrophoresis is similarly linear. This type of misinformation is unhelpful whatever the intended target audience of the book. Section 47 (Electrophoresis of Nucleic Acids) does nothing to put right this incorrect notion of fragment separation. Indeed, it introduces confusions of its own by erroneously grouping pulsed-field gel electrophoresis (PFGE) with denaturing-gradient gel electrophoresis (DGGE) and temperature-gradient gel electrophoresis (TGGE). The, incorrect, impression given is that PFGE results in the denaturation of duplexed DNA. In addition, the depiction of agarose gel electrophoresis in the upper part of Fig. 47.1 is not an ideal aid to an understanding of the process. The suggestion in the compass box in Section 44 that the modern routine method for the detection of the mutation causing sickle-cell anaemia is Southern-blot hybridization is, again, misleading. Although it clearly can be done in this way, polymerase chain reaction (PCR) amplification offers a superior technological approach.

From my personal perspective, the omission of Mutation Detection as a section in its own right is a serious flaw. Such a section could have been home to mutation-scanning methods such as PFGE and TGGE and the simpler, and much more commonly used, single-strand conformation polymorphism method, which does not appear to be mentioned anywhere in the book. Mutation detection by chemical cleavage of mismatch is similarly absent. The use of RFLPs, the amplification-refractory-mutation system and allele-specific primers for the detection of known mutations would also be natural residents of such a section.

Noting that there was a section dedicated to 'Detecting DNA–Protein Interaction Sites', I expected to find a section on the subject of protein–protein interactions identified using the yeast two-hybrid (interaction-trap) system. No such luck. There may be discussion of the elegant power of the two-hybrid system somewhere in the book, but I can't find it.

It's disappointing to find, in Section 14, an error that I have heard made numerous times by undergraduates, who confuse signals for the termination of transcription with translation termination codons. Such a fundamental error is inexcusable. Undoubtedly, I have been selective in the choice of sections that I have carefully reviewed, and perhaps others approaching this book from a different perspective would find fewer errors – but I'm not so sure.

Although the subject matter of the book is copiously referenced, the three bibliographies at the end of the book are of little use in themselves. The references do not appear to be sorted in any way and each begins with the title of the work rather than the first author's name. An opportunity has been missed to provide something more useful.

I'm not even convinced, in spite of what the authors claim in the introduction, that the book delivers in terms of the concept of 'Route Maps'. Rather than providing maps, it's more like a gazetteer and photo album. When I first received this book for review I was immediately enthused by what it was attempting to do, but now enthusiasm has been replaced by despondency at an opportunity missed. Using the book is rather like asking for directions to somewhere and being told, 'Well, I wouldn't start from here.'

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The curate's egg

Cereals: Novel Uses and Processes

edited by G. M. Campbell, C. Webb and S. L. McKee, Plenum Press, 1997. US$95.00 (xvi + 289 pages) ISBN 0 306 45583 8

The conjunction of overproduction of cereals (particularly wheat) with growing social and environmental pressures to reduce depletion rates of fossil reserves has pushed the consideration of novel uses for cereals higher up the agenda. This volume is a compilation of papers presented at an international conference held in June 1996; the editors and publishers should be congratulated for bringing the proceedings into the public domain in such a (relatively) short time.

The material has been divided into three sections, following the structure of the conference sessions. The first deals with the individual components of cereals – 'starches', 'proteins' and 'bran and straw' (an odd combination!); the second with whole-crop utilization, predominantly fermentation; and the final section with processes explicity to do with food. As is inevitable with conference proceedings, the result is less than balanced, reflecting the topics of the individual papers submitted rather than the product of an editorial policy aimed at holistic coverage, and, just as inevitably, the quality of the material varies from the highly informative to the anodyne, and from the very specific

to the rather general. This results in a lack of focus – the material jumps between topics with bewildering rapidity, in a way one expects more with a research journal than a book.

The section on starches, which comprises by far the largest individual part of the book, suffers the least from the lack-of-focus problem. For the peripheral reader, here at least is room for some development of specific topics, such as the use of starch as a feedstock for the production of plastics and chemicals. However, one still finds odd topics juxtaposed – descriptions of mathematical modelling of the production of lactic acid from starch by fermentation next to a discussion of the derivatives of levulinic acid, an acid-hydrolysis product of various carbohydrates. For a highly specific topic, it is hard to beat the description of the effect of corn-starch extenders on the comfort factor of polyurethane foams used for padding furniture – interesting, but only of minor significance as an end-use, if one follows the earlier discussions of industrial markets for starch. The remaining sections in the 'cereal components' part of the volume are small and diverse. Proteins contribute around 10% of the dry matter of a typical cereal grain and are potentially a high-value product, but they have not attracted much interest in these proceedings. Similarly, bran and straw have been thrown together, although they are hardly related in a biological sense.

Mature cereal straw (annual UK 'production' 11.5 million tonnes) is usually regarded to be an unfortunate byproduct, but it represents about half of the above-ground biomass of the crop. Its potential as a sustainable fuel is enhanced, at least in the UK, by government policy aimed at encouraging nonfossil-fuel usage. As described in this volume, it can also serve as a feedstock for the production of ethanol and other industrially useful organics. Nevertheless, its exploitation is limited, as reflected by the few contributions in this volume, largely due to adverse financial returns to the producer and/or taxpayer, and a negative global CO_2 balance (given the high fossil-fuel requirements of modern farming technology). Whole-crop fermentation, on the other hand, may be more a more promising field, and various aspects are described in a short series of papers.

The final section of the book deals with some aspects of food-processing technology. This is a highly diverse topic, given the plethora of processed food products that are made using cereal raw materials. It would have been useful for the general reader to preface this section with an overview of this product disparity, in a similar way to that in the earlier section on the industrial usage of starch. In the absence of this, the section has a disjointed feel and lacks cohesion.

Overall, this is a finished-looking book that does not appear, as do so many conference proceedings, to be a compilation of disconnected papers, each with their own editorial and typographical style; however, the extent of editorial control over its content is not so clear. Thus, for example, although the conference was dedicated to the exploration of novel uses for cereals, with the accent on the future, there is, surprisingly, no mention of the potential of transgenic cereals as a vehicle for the production of high-value molecules. My overall impression of the book is that it is a curate's egg – good only in parts.

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Guessing protein structures

Protein Structure Prediction: A Practical Approach

edited by M. J. E. Sternberg, IRL Press at Oxford University Press, 1996. UK£27.95 (xix + 297 pages) ISBN 0 199 63496 3

As you walk away from a structural biology meeting, you might feel that reliable protein-structure prediction is almost possible and just a matter of getting the right software. Clearly, there is a need for a book to tell you how realistic this is and, of course, it is not realistic in the most general sense, but there are many subsets of the problem and this book is split into chapters describing some of them.

The better chapters are very realistic and helpful. King's chapter on secondary-structure prediction is objective and should leave you with no illusions – you know from the start that you are going to be wrong 25–35% of the time. The chapter by Jones *et al.* really has two parts: there is first an excellent overview of protein folds, followed by an introduction to knowledge-based force fields and their use in recognizing the most probable fold for an amino acid sequence. The best chapter may well be Shoichet's discussion of predicting protein–ligand complexes. He is probably the only author to subdue his preferences and give a real overview of what software is available. There is also a good list of when things are likely to break, mislead and waste computer time.

Each of the authors writes about their field of expertise, but there is some overlap and duplication. For example, Srinivasan *et al.* provide a well-referenced list of diagnostics to check when assessing a protein model (stereochemistry, appropriate solvent exposure and shielding, results from knowledge-based force fields, etc.). This list is repeated three chapters later, but with less references and under the title 'Theoretical constraints on model structures'. Some harsh editing could have removed the second treatment and replaced it with a reference to the first. It also seems that authors never saw each other's work. The 'Combinatorial Approach' chapter refers to the existence of a chapter on secondary-structure prediction, but then briefly reviews the topic from their perspective. The chapter on secondary-structure prediction has a section entitled 'The Cohen method', but does not refer to Cohen's chapter in the same book.

There is an assumption in the structure of the book that you have some idea about the applicability of

the methods and their reliability or precision. Sometimes this is clear; if your sequence of interest has more than 90% homology to something of known structure, then you would go straight to the chapter by Srinivasan *et al.* on homology modelling. If, however, you have 20–25% sequence homology, it is less clear where you should go. You should probably be sent away to study Barton's chapter on database searching before moving on. Alternatively, you might go straight to the chapter on protein-fold recognition, but will you get a feeling for what kind of success you can expect? The modelling chapter quotes root-mean-square (rms) differences in Ångstroms, but how does this compare with the structural homology between members of a single protein-fold category? Perhaps one should abandon hope of a 3D structure and just try to find some motif that may tell you about the function; or perhaps this is not the right approach and you should first read the chapter on membrane-spanning proteins to see if your protein isn't really soluble and globular after all. It probably would not do this book any harm to have some overview and discussion of when to use the different methods.

In any lively field, a book will start to age before it leaves the printer's warehouse. This work suffers from this in some respects. Hidden Markov models are becoming popular for (multiple) sequence alignment. They deserve more space and perhaps comparison with more-established algorithms. The biggest omission is probably in the chapter on transmembrane protein topology – after reading this, you would still have no idea about the work of Rost or Argos, which is a pity, because they claim more than 85% correct performance.

It is not reasonable to expect a small 'practical approach' to cover every possible topic, and there are some small gaps. True *ab initio* structure prediction is still a distant goal, either on lattices or in continuous space, but it is a very popular area. Perhaps the editor felt that the world did not need another review comparing devious search algorithms and simulated annealing, but it might have been worth covering this topic briefly in one of the other chapters.

The highlights of this book make it worth buying, and perhaps a future edition with more coordination between the authors will strengthen it even further.

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Everything you ever wanted to know about biotransformations...

Biotransformations in Organic Chemistry

by Kurt Faber, Springer, 1997. US$44.95 pbk (x + 406 pages)
ISBN 3 540 61688 8

In this, its third edition, *Biotransformations in Organic Chemistry* establishes itself further as a must for the bookshelf of any organic chemist involved in research or product development. The same tried-and-tested format that was fine-tuned in the first two editions is also evident in the new edition. The book represents a summary of the literature up to the end of 1996 that pertains to the application of both enzymes and whole-cell microorganisms to organic synthesis. The chapter sizes reflect the significance of the impact of biocatalysis, and include hydrolytic and oxidation/reduction reactions as well as those forming carbon–carbon bonds.

The explosion of literature concerned with biocatalysis over the past decade makes reviewing this area in a single volume extremely difficult, but Faber manages to achieve this in a style that is both logical and reader-friendly. The clarity with which he explains the mode of action of various enzymes means that it should be read by the many chemists who still see this as 'black-box' technology.

In such an ambitious venture, it is inevitable that certain developments have been understated. For instance, metabolic-pathway engineering (particularly the shikimate pathway) to produce high-value intermediates from cheap, renewable input substrates such as glucose is not mentioned. The forced evolution of microbial strains to produce 'designer catalysts' is another rapidly developing area that has been neglected.

The accuracy of the information relayed in the book is generally very high. The only inaccuracy I found in the text was early in the book, where Faber quotes the Diels–Alder reaction as one for which there is no natural equivalent; microorganisms that catalyse these reactions have, in fact, recently been reported. There are, however, significant inaccuracies in Appendix 5.3, which lists the commercial suppliers of lipase preparations. There are several instances in which these companies no longer supply the lipases accredited to them; indeed, some of the lipases listed now have, to my knowledge, no commercial suppliers. It is also suggested that *Pseudomonas fluorescens* lipase has been renamed *Pseudomonas cepacia* lipase; this is not the case, for they are distinct (albeit closely related) species.

These minor blemishes in no way detract from the merits of this book, which is unquestionably the best text currently available for those looking to exploit the potential offered by biocatalysis.

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Biochemical engineering – past, present and future

Biochemical engineering is 50 years old! The first recognizable biochemical engineering process emerged in 1947 with the large-scale production of streptomycin (by Merck). Since then, biochemical engineers have played a pivotal role in the translation of bioscientific discoveries into biotechnological products. Today, biochemical engineers are not only working on the design and scaling up of bioreactors, but also in areas such as drug discovery and biocatalyst engineering, which require expertise in molecular and cellular biology, biomaterials and combinatorial chemistry. The aim of a recent conference organized by W-S. Hu (University of Minnesota, Minneapolis, MN, USA) and J. Swartz (Genentech Inc., San Francisco, CA, USA)* was to celebrate the diversity of modern biochemical engineering and identify its role in the future development of biotechnological products and processes. In this report, I have focused on a number of papers that give a flavour of the new and innovative developments in biochemical engineering; abstracts of all the oral and poster presentations are available (http://www.cems.umn.edu/~wshu_grp/efc/efc.html).

The fields of biochemical engineering and molecular biology have been converging for several years, and it is now commonplace for biochemical engineers to use molecular-biological methods as research tools or develop new genetic methods to improve process productivity. J. Keasling (University of California, Berkeley, CA, USA) discussed the development of new tools that are required for successful metabolic engineering. For example, the use of stable, low-copy-number plasmids such as the F plasmid of *Escherichia coli* has advantages for protein expression, because they replicate in tandem with cell division, thus ensuring that each daughter cell receives a copy of the plasmid. In another approach, the control of gene expression was improved by the use of a cassette system designed to optimize mRNA stability against degradation by introducing a 5′ RNA hairpin into the mRNA. This gave a threefold increase in mRNA stability, which subsequently improved protein production approximately fourfold.

The current antibody market is estimated at US$1–1.5 billion, consisting mostly of diagnostic products, but the ultimate challenge is to mimic and improve upon the mammalian immune system. G. Georgiou (University of Texas, Austin, TX, USA) and D. Wittrup (University of Illinois, Urbana, IL, USA) discussed methods for the molecular engineering of antibodies that allow high binding affinities to be produced to order. G. Georgiou presented two techniques: *in vitro* scanning saturation mutagenesis and fluorescence-activated cell sorting of antibodies expressed on the surface of *E. coli*. D. Wittrup has a similar approach to engineering protein recognition, but based on the surface display of antibodies on yeast. Using yeast as the display support has the advantages of possessing compatibility with eukaryotic expression, leading to elimination of stochastic effects and permitting the use of soluble antigens.

The phenomenon of protein aggregation is important in many bioprocesses, for example crystallization, drug formulation, inclusion-body formation and protein refolding. H. Blanch (University of California, Berkeley, CA, USA) described a molecular-thermodynamic model combining physicochemical data and statistical mechanics to describe protein precipitation; the advantage of this model is that it is based on the major molecular forces implicated in protein–salt interactions, enabling accurate predictions to be made. This paper was included in a wide-ranging session on unit operations that included reports on the status of bioreactor mixing (A. Nienow, University of Birmingham, Birmingham, UK), the effect of fluid forces on sub-lytic effects on cell culture during scale up (M. Shuler, Cornell University, Ithaca, NY, USA) and the optimization of perfusion-culture conditions based on an economic model (K. Konstantinov, Bayer Corp., Berkeley, CA, USA).

It is likely that, in the future, plant maintenance will be achieved with the use of robots. This was illustrated by I. Endo (RIKEN, Saitama, Japan), who has developed three types of autonomous robot to carry out specific tasks in bioprocess plants: vision, transport and manipulation. Functioning through a distributed control system, this system was shown to offer a flexible and robust mediator between the plant operator and the process. Other reported advances in product analysis, process monitoring and control included reactor and product monitoring using 2-D fluorescence (T. Scheper, Institute for Technical Chemistry, Hannover, Germany) and the industrial use of 'electronic nose' technology for monitoring contamination and assessing consistency during fermentation processes (P. Namdev, Schering-Plough, Union, NJ, USA).

The biochemical engineering of plant products has traditionally been confined to *in vitro* plant-cell culture, but the use of plants in biotechnology is not confined to sources of bioproducts, as is seen increasingly in the use of transgenic plants as expression hosts for recombinant proteins (Z. Nikolov, Iowa State University, Ames, IA, USA). This field has expanded to include the production of commodity chemicals such as organic acids from the fermentation of plant raw materials (P. Elankovan, MBI International, Lansing, MI, USA), bioprocessing in tree production (R. Timmis, Weyerhaeuser Co., Tacoma, WA, USA) and bioprocessing in the agricultural-processing industry (D. Johnson, Grain Processing Corp., Muscatine, LA, USA).

The development of medical devices requires interdisciplinary skills, including the use of biomaterials, quantification of drug transfer and clearance rates and enzyme immobilization. This area is full of activity by biochemical engineers. D. Mooney (University of Michigan, Ann Arbor, MI, USA) is researching synthetic extracellular matrices to control tissue structure. The use of cell-adhesion peptides covalently coupled to alginates gives an immobilization system that allows specific recognition and adhesion. Using hepatocytes as a model, the incorporation of the adhesion peptides was shown to enhance cell proliferation and differentiation. The surface chemistry of a material can be engineered with biomolecules to

*Biochemical Engineering X, Kananaskis, Alberta, Canada, 18–23 May 1997.

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PII: S0167-7799(97)01097-4

resist nonspecific protein binding or cell adhesion (K. Healy, Northwestern University, Chicago, IL, USA). For example, the covalent modification of a polymeric network of polyacrylamide and polyethylene glycol with a bioactive peptide from the cell-binding domain of bone sialoprotein was shown to support cell attachment and proliferation, which did not occur in its absence. Other papers in this session reported the development of a bone-marrow culture system that mimics the *in vivo* marrow structure to stimulate haemopoiesis (D. Wu, University of Rochester, Rochester, NY, USA), the development of a controlled-release polymer that, in contact with mucosal sites, enables topical immunoprotection with monoclonal antibodies (M. Saltzmann, Cornell University, Ithaca, NY, USA) and the development of an immunomagnetic cell separator that enables cells to be fractionated into subpopulations based on the density of the active cell-surface receptor (J. Chalmers, Ohio State University, Columbus, OH, USA).

The production of biopharmaceuticals remains central to biochemical engineering. Advances in process development include new stationary phases for chromatography (M. Ladisch, Purdue University, West Lafayette, IN, USA), the use of displacement chromatography for protein purification (S. Cramer, Rensselaer Polytechnic Institute, Troy, NY, USA) and the characterization and improvement of protein-refolding processes (E. De Bernardez-Clark, Tufts University, Medford, MA, USA). New process tools were illustrated – for example, how the use of cell-cycle analysis can enable the prediction of culture performance to aid scheduling of cell culture reactors (M. Croughan, Genentech Inc.). J. Swartz (Genentech Inc.) described how host-cell cross-contamination can be detected in multiproduct facilities by using negative genetic markers based on nutrient utilization. Many aspects of protein formulation were also discussed at this meeting, ranging from an analysis of protein degradation during processing (R. Borchardt, University of Kansas, Lawrence, KS, USA) through a study of formulation operations (J. Cleland, Genentech Inc.) to the development of a new microsphere–protein delivery system (M. Tracy, Alkermes Inc., Cambridge, MA, USA).

As biochemical engineering develops in parallel with other advances in the biosciences, a number of new areas will emerge, and several such topics were discussed. J. Keller (SmithKline Beecham Pharmaceuticals, King of Prussia, PA, USA) described advances in drug discovery using genomics and combinatorial chemistry based on nanoscale ultra-high-throughput screening, and the associated developments in informatics that will allow structure and function to be determined from primary gene-sequence data. The use of biotransformation in the development of a method to protect military personnel against the effects of organophosphate nerve gas was presented by K. LeJeune (University of Pittsburgh, Pittsburgh, PA, USA). This method centres on the immobilization of phosphotriesterase (an organophosphate hydrolase) onto polyurethane to be used as a selective barrier against nerve agents. In laboratory tests, this system showed active and stable performance in a continuous reactor at 75°C for 24 hr. A new development in combinatorial chemistry was presented by D. Clark (University of California, Berkeley, CA, USA) – the use of libraries of enzymes acting on a single compound, allowing a combinatorial-biocatalysis approach to the generation of new compounds. This offers the advantages of high regio- and stereoselectivity, one-step reactions and mild reaction conditions, and, in addition, all the reactions are biologically relevant, and this increases the chances of identifying new pharmaceutical lead compounds.

The past and future status of biochemical engineering are best summarized by the three keynote lectures given at this conference. A. Humphrey (Penn State University, University Park, PA, USA) described the origins and early years of this discipline in terms of the scale-up and operation of industrial submerged-culture fermentation processes that required innovations in heat transfer and mixing operations. J. Bailey (ETH, Zurich, Switzerland) reviewed the use of mathematical modelling and analysis in biochemical engineering. This is perhaps the unifying theme in both process and molecular-biochemical engineering; the necessity for and ability of biochemical engineers to provide a quantitative description of both engineering and biological phenomena. Finally, D. Wang (MIT, Cambridge, MA, USA) noted that the recent trends in biochemical engineering have been to move away from equipment design, to take a less macroscopic view of bioprocesses and to increase the emphasis on modern biology and cellular processes. The future for biochemical engineering research will inevitably be in fields such as rational drug design, biocatalysis, combinatorial chemistry and the development of new small-molecule drugs such as the next generation of antibiotics.

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The **Forum** section of *TIBTECH* is a platform for debate and analytical discussion of newly reported advances – whether in the research literature or at conferences. It includes *Workshops, Meeting reports,* and *Letters to the Editor*.

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Biocatalysts – the next generation

The discovery of new catalysts and molecules that rival natural enzymes in their substrate specificity and catalytic turnover is of major importance in the production of compounds for pharmaceutical use, in the development of novel biosensors and in the *in-vivo* use of such molecules to treat human disease. In addition to these practical considerations, the insights into the functioning and organization of catalytic biomolecules in cellular development and reproduction help to answer a number of key questions in biology.

Present approaches to the creation of novel catalysts fall into two general categories – the *de novo* design and synthesis of catalysts from organic molecules such as macrocyclic compounds, polymers, cyclodextrins and peptides, and the modification of existing catalysts, such as enzymes or ribozymes, by genetic or chemical methods. 'Abzymes' can be considered to be a hybrid of the two, as their production involves using molecular design and synthesis to create a transition-state analogue, which is then used to tap the immunological response for antibodies that bind to it and are thus potential catalysts for the reaction involving that intermediate. Advances in catalyst production include the construction of ribozymes capable of cleaving a viral-DNA target, the induction of catalytic antibodies that promote stereospecific ester and peptide synthesis or unfavoured chemical reactions, the formulation of enzymes to function in organic solvents, and the modification of enzymic activity to broaden substrate specificity. These advances have been associated with equally valuable 'spin-off' technology, for example, the cloning and expression of antibody fragments in bacteria, improvements in solid-state peptide synthesis and structural computer modelling.

These and other recent advances set the stage for examining a number of key issues (e.g. the minimum size for a catalyst, the importance of long-range molecular forces for substrate specificity and optimal turnover, the development of computer methods with predictive capabilities, the definition of novel medical targets within the scope of available catalysts and the applications of such catalysts to organic synthesis) in a recent symposium* that provided opportunities for interdisciplinary researchers to discuss many of these issues.

Enzymes

This session provided an opportunity for examining the catalytic efficiency of enzymes, relative to the spontaneous reaction, for a given process. The range of catalytic efficiencies of enzymes has proved to be considerable, from a factor of 10^6 to one as high as 10^{24}. Interestingly, the rates of reactions catalyzed by enzymes for these processes are very similar to that required by the network of reactions in a living organism (R. Wolfenden, University of North Carolina, Chapel Hill, NC, USA). The way these marvellous machines evolved from ancestral families appears to be within our grasp, now that powerful algorithms allow the reconstruction of the primordial genes and, through recombinant techniques, expression of these proteins and the study of their catalytic function (S. A. Benner, University of Florida, Gainsville, FL, USA). There are still a large number of organisms that have not been screened and examined for their ability to catalyze specific reactions, and, with the ever-increasing size of genomic databases, the range of reactions catalyzed by enzymes will certainly expand. Consequently, powerful screening techniques (P. Cheetham, Zylepsis, Ashford, UK) need to be developed for exploring both the nature of enzymes in various organisms and also the properties of recombinant enzymes that are isolated from combinatorial libraries. Moreover, enzymic catalysis is no longer restricted to aqueous media; there is considerable evidence that enzymes function in nonaqueous media, in which their turnover and stereospecificity can be manipulated (A. M. Klibanov, MIT, Cambridge, MA, USA).

Engineered enzymes

Protein engineering, by either recombinant-DNA methods or chemical means, holds considerable promise for extending the stereospecificity and regioselectivity of many enzymes. Recognizing that proteins can often be divided into discrete domains, the possibility of swapping these domains to create novel catalysts with changed substrate specificity or reaction characteristics seems to be within reach. Many of these changes will be irrational and unpredictable, given existing structural databases: their discovery will require combinatorial libraries. The resulting catalysts may be composites between naturally occurring amino acids and other organic reagents that can be introduced either by synthetic or recombinant methods.

Following this approach, A. Ballesteros and F. J. Plou (CSIC Institute of Catalysis, Madrid, Spain) presented data on semisynthetic enzymes obtained by chemical modification with fatty acids or polyethylene glycol. R. Offord (University of Geneva, Geneva, Switzerland), in contrast, dealt with semisynthetic proteins fabricated by linking the chain termini of peptides and proteins. The engineering of a cofactor-binding domain to convert its coenzyme specificity from NAD to NADP (R. N. Perham, University of Cambridge, Cambridge, UK) and of the carboxypeptidase protein inhibitor to smaller, capped peptides (F. X. Avilés, Universitat Autònoma de Barcelona, Bellaterra, Spain) proved fruitful. Other work presented included the engineering of subtilisin for the specific cutting and splicing of proteins (J. A. Wells, Genentech Inc., San Francisco, CA, USA). Structures based on α-helical models also proved effective at forming ion channels (W. F. DeGrado, University of Pennsylvania, PA, USA). S. M. Hecht (University of Virginia, Charlottesville, VA, USA) reported changes in the catalytic behavior of dihydrofolate reductase and luciferase that contained synthetic amino acids at defined positions. Molecular imprints (K. Mosbach, Lund University, Lund, Sweden) created from polymeric materials were reported to allow the formation of artificial binders with altered substrate specificity.

Catalytic mechanisms

A fundamental tenet of enzymic catalysis is the principle of transition-state stabilization, which underlies many of the explanations for an

* The Juan March Foundation Workshop on 'Novel Biocatalysts' was held in Madrid, Spain, 10–12 March 1997.

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PII: S0167-7799(97)01098-6

enzyme's superior catalytic efficiency when compared with chemical catalysts. Reexamination of this issue by model systems (A. J. Kirby, University of Cambridge, Cambridge, UK) and computational methods suggests that some of these beliefs should be tempered. For example, computational methods suggest that the ground-state enzyme–substrate complex may be in a more enthalpically reactive conformation than originally thought, providing an impetus for the examination of such species by a variety of spectroscopic methods (T. C. Bruice, University of California, Santa Barbara, CA, USA).

The complexity of enzymic catalysis has always been subjected to dissection in a variety of model studies. Those involving supramolecular complexes, which follow enzyme-like kinetics in processing their substrates, may provide valuable insights into the importance of balancing rigidity and dynamics in the catalytic process. J-M. Lehn (Université Louis Pasteur, Strasbourg, France) explored supramolecular reactivity and catalysis, while J. de Mendoza (Universidad Autónoma de Madrid, Madrid, Spain) presented his latest results on the formation of stable capsules, in whose interior reactive species can be accommodated.

Ribozymes and abzymes

Ribozymes and abzymes have now appeared on the scene as another form of biological catalyst, the former being naturally occurring and the latter manmade. Key questions include whether these catalysts can be manipulated to improve their catalytic efficiencies, and, perhaps more importantly, what they can tell us about enzyme function.

The differences between RNA and protein enzymes helps to teach us about the aspects of RNA and proteins that are distinct, whereas the common features help us to understand the aspects that are fundamental to biological catalysis (D. Herschlag, Stanford University, Stanford, CA, USA). O. C. Uhlenbeck (University of Colorado, Boulder, CO, USA) discussed the interaction of ions with hammerhead ribozymes and how to develop solution methods to locate functionally important metal-ion binding sites and relate them to the crystal structure.

Comparing enzymes and abzymes, it is useful and rewarding to attempt to construct a catalyst not only for practical purposes but also to improve our understanding of the principles underlying catalysis. S. J. Benkovic (Pennsylvania State University, University Park, PA, USA) developed, through a unique hapten design, a catalytic antibody able to form various dipeptides with all possible stereochemistries at C_α. Natural enzymes have evolved over millions of years; multiple cycles of mutation, selection and amplification can also be exploited in the laboratory to create and characterize protein catalysts on a human time scale. This provides access to macromolecules with tailored activities and selectivities (D. Hilvert, The Scripps Research Institute, La Jolla, CA, USA).

Given that catalysis cuts across so many scientific disciplines, this meeting was particularly important, because it brought together a number of investigators who view this subject from different aspects. Some were grounded in the practicality of finding enzymes or catalysts for specific applications, others more interested in the fundamentals of how enzymes function or how they evolved, and still others were making a strong effort to build catalysts using synthetic or combinatorial approaches.

As for future trends, we see a strong influence by enabling technologies, including: (1) larger arrays for a given platform (e.g. phage display); (2) increased complexity in a given platform (e.g. modular design); (3) information from databases (e.g. sequence and structure); (4) clarification of the fundamentals (e.g. dynamics, femtosecond measurements). In addition, a general trend to increased complexity in model systems is anticipated (with a balance between rational and random approaches). In the next few years, important discoveries in several areas (e.g. novel spectroscopies and surface chemistry) will further our knowledge.

The rational use of current enabling technologies (e.g. mutagenesis, chemical modification, solvent effects, molecular design and computation, genetic sequences and combinatorial phage display), thoughtfully exploited in various model systems (structural α-helices, imprinted templates, macromolecular domains, enzymes, ribozymes and abzymes), will make it possible to increase our present knowledge of basic and applied aspects of novel biocatalysts.

All the participants at this conference agreed that the experience of finding a common language for communication, as well as the opportunity to discuss issues in depth, made this meeting both valuable and memorable.

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Letters to the Editor

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USDA regulation of field trials of recombinant-DNA-modified plants: reforms leave severe flaws

In May 1997, the United States Department of Agriculture (USDA) announced a reduction in the regulatory requirements for field trials involving recombinant DNA (rDNA)-modified plants[1]. In the announcement, the agency responsible for this regulation, the Animal and Plant Health Inspection Service (APHIS) states that it 'anticipates that this Final Rule will be generally welcomed by public and private researchers because it is estimated that it could save the industry as a whole perhaps $50,000 in costs associated with preparing submissions'[1].

A decade earlier, USDA had created those regulations[2,3], requiring for the first time in the history of plant breeding that field trials with new cultivars and breeding lines could proceed only under a federal-permit and environmental-assessment system. The regulations applied only to certain plants that had been genetically modified using recombinant DNA techniques (defined as 'regulated articles'). The announced changes will reduce the burden of the permit-application process by enabling researchers to use a streamlined notification system. USDA has been piloting the notification process since 1993, when the regulations were first modified for a limited number of specific commodity crops: corn (*Zea mays*), cotton (*Gossypium hirsutum*), potato (*Solanum tuberosum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicum esculentum*).

The notification process reduces the time spent in preparing permit applications and enables researchers to launch field trials in a more timely manner. This is good news for academic researchers, who typically have no regulatory-affairs staff to assist in the preparation of the detailed application documentation and who must use research grants to cover the expense of regulatory compliance.

As we have reported elsewhere[4], the benefits of the notification process have to date been largely restricted to the private sector (Fig. 1). Companies, especially the largest (such as Monsanto, Pioneer and Ciba Geigy) continued to focus their R&D efforts heavily on the six crops that became eligible for the notification process under the 1993 amendment (Fig. 2a). USDA regulation has placed a special burden on academic institutions and young, entrepreneurial businesses. Researchers in these sectors are generally less well funded and yet they have continually borne much higher regulatory costs, simply because they study a broad array of crop species (Fig. 2b).

The expansion of the notification process to the full spectrum of common crop species means that academic researchers and smaller companies can now devote more time and a greater portion of their limited research funds to conducting research. With this change, USDA signals that case-by-case environmental assessment by the federal government is not necessary for conducting field trials safely for the vast majority of rDNA-modified plants; notification of the agency is sufficient. This change not only reduces the burden on applicants but should also have a substantial effect on reducing the efforts required at APHIS.

USDA has taken a step in the right direction, but the impacts of the expanded eligibility for notification will not be as great as claimed by the regulators. APHIS continues to impose unnecessary and hugely expensive field-test-design and reporting requirements that impose a greater burden on researchers than even the permit-application process. Because the design, monitoring, record-keeping and reporting requirements are unchanged under the notification system, the actual time and financial savings to applicants are substantially less than they could and should be. In a 1995 Memorandum, President Clinton directed heads of departments to consider the question: 'Could private business, setting its own standards and being subject to public accountability, do the job as well?' In other

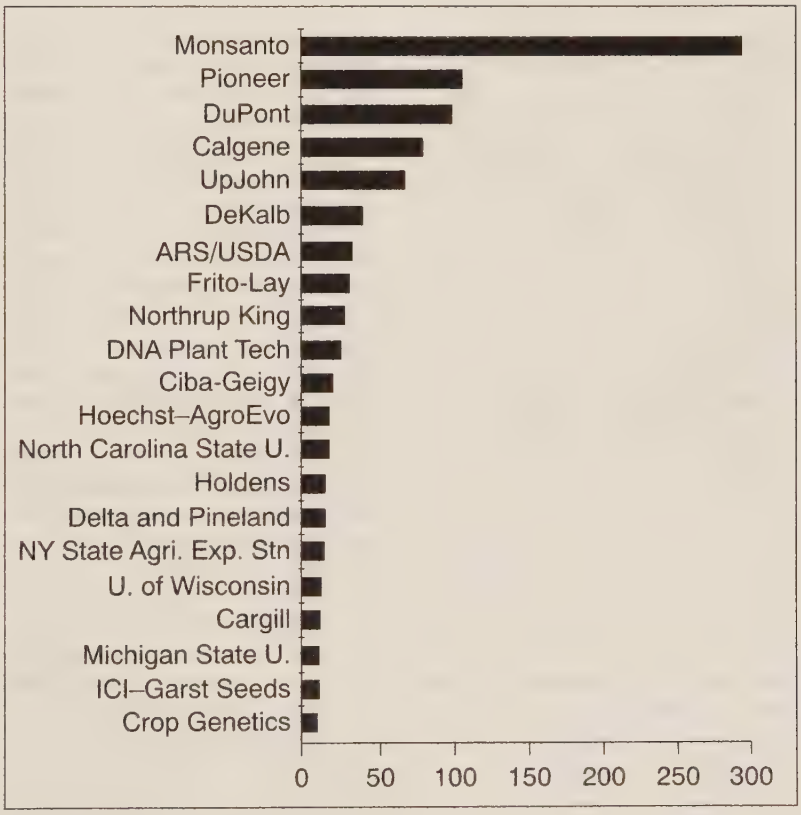

Figure 1

The number of field-trial permits and notifications by the US Animal and Plant Health Inspection Service to specific applicants between the start of 1987 and 30 September 1994. Reproduced with permission from Ref. 10.

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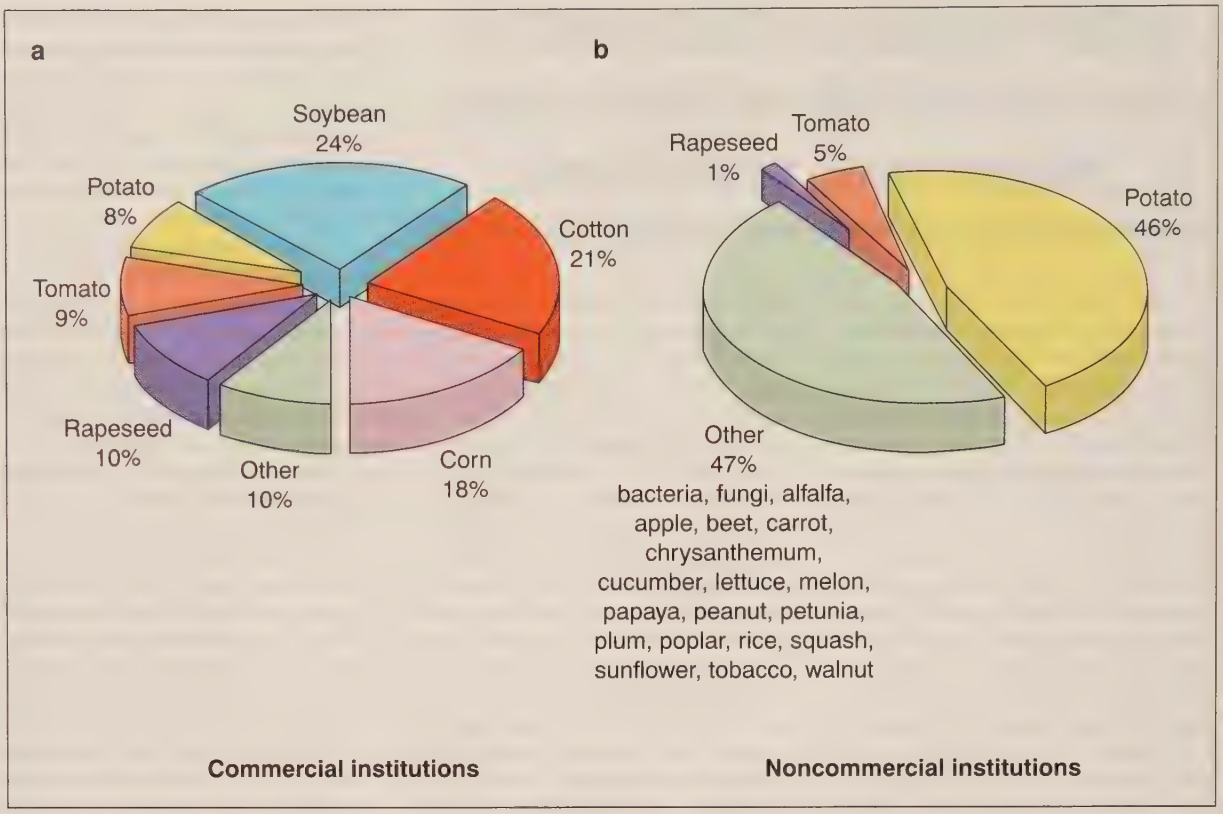

Figure 2

The proportions of field-trial permits and notifications approved by the US Animal and Plant Health Inspection Service for specific crops to commercial (**a**) and noncommercial (**b**) institutions between the start of 1987 and the end of May 1994. Reproduced with permission from Ref. 10.

words, could the community of plant-breeding researchers establish adequate standards and procedures to ensure the safe field testing of rDNA-manipulated organisms? In our opinion, the answer to this is certainly 'Yes'. (It is noteworthy that the oversight of dangerous pathogenic microorganisms in US laboratories – including those that cause diseases such as anthrax, bubonic plague and Ebola fever – is governed by voluntary guidelines promulgated by the Centers for Disease Control and the National Institutes of Health[5].)

USDA seems to agree, to some extent. In the announcement, APHIS refers repeatedly to the effectiveness and appropriateness of routine agricultural practices adopted for conventionally bred crop varieties as generally adequate for controlling gene flow from rDNA-manipulated plants. APHIS regulators are also well aware of the widely held consensus that neither gene splicing nor the movement of genes between distantly related organisms *per se* confers additional risk. There is no evidence, however, that APHIS intends to evolve its regulations towards eliminating the discrimination against rDNA-manipulated organisms.

USDA needs to take a hard look at the continuing rationale behind its performance standards, so-called. They are actually highly prescriptive design standards, limited to the single goal of stringent confinement, rather than being intended to identify field-research procedures that provide a level of control that is commensurate with the level of risk involved in the trial. The USDA's recurring assertion that the 1997 'rule is consistent with the risk-based and product-based philosophy underlying the Federal policy for the regulation of the products of biotechnology'[1] is simply untrue. It is neither risk-based nor product-based, nor has any USDA regulatory policy toward transgenic plants during the past decade satisfied these criteria. The regulations continue to focus solely on organisms modified using rDNA techniques and rely on the fiction that rDNA-manipulated plants present a plant-pest risk when they contain DNA sequences (i.e. vector or regulatory sequences) derived from plant pests. The concept of the 'regulated article' has no scientific foundation and needs to be refocused clearly on risk.

USDA should introduce oversight based on genuine performance standards. The agency's current 'performance standards' prescribe rigid requirements for all aspects of field research, from the shipping of transgenic material to the final disposition of plants, regardless of the actual level of risk that may derive from invasiveness, gene flow, persistence in the environment or ecological interactions. In contrast, genuine, outcome-based performance standards would result in a spectrum of approaches and control levels dictated by the actual levels of risk involved. By introducing a bona fide performance-standard system, USDA would reduce the waste of limited research funds on extreme confinement measures when less costly and intrusive approaches would suffice.

USDA should take into consideration the safety record of plant breeders and the fact that millions of new genetically modified plants are field tested each year without governmental oversight of any kind. (The average plant breeder of corn, soybean, wheat or potato, for example, may introduce 50 000 genotypes per year into the field, and these may be the product of 'wide crosses', in which genetic material has been transferred via hybridization across

species or genera.) Add to this the absence of any reports of adverse outcomes in any of the more than 1500 field trials of rDNA-manipulated plants overseen by the APHIS permit system. The vast majority – perhaps all – would have been adequately controlled with standard plant-breeding practices. Had they been subject merely to these standard agricultural practices, small agbiotech businesses and academic researchers could have directed substantially greater amounts of their limited research funds to research, and their projects would thus have advanced considerably further.

In conjunction with a permit system to accommodate field trials with organisms of known or suspected serious risk[6], an oversight system built on professional plant breeders' standards is likely to be highly effective for work with rDNA-manipulated plants. It would have the advantages of familiarity and broad acceptance within the regulated plant breeders' community and the added strength of government regulation for cases of greater potential risk.

Judging from USDA's arguments in the May 1997 announcement, however, the fate of further regulatory reform continues to rely less on arguments about public and environmental safety than on the agency's perceived need for public relations. 'The availability of field trial reports, including the vast majority not reporting unexpected events, may help to increase public confidence about the conduct of field trials. For this reason we believe that there is significant benefit in maintaining reporting requirements for all field trials under notification or permit procedures at the present time.'[1]

USDA's amorphous intention to 'increase public confidence' hardly constitutes justification for continuing to over-regulate biotechnology, with its attendant direct and indirect costs. Moreover, such over-regulation arguably has exactly the opposite of the intended effect. Consider this observation by the president of a consumer advocacy group: 'For obvious reasons, the consumer views the technologies that are most regulated to be the least safe ones. Heavy involvement by government, no matter how well intended, inevitably sends the wrong signals. Rather than ensuring confidence, it raises suspicion and doubt.'[7]

Another defect in USDA policy making is its reliance on direct experience with field trials – no matter how redundant or devoid of empirical value – rather than accepting the widely held consensus of the scientific community about the continuum that exists between 'conventional' and 'new' biotechnology[8]. Academic and commercial researchers have patiently cooperated with USDA for nearly a decade. Databases filled with redundant findings of 'no adverse effect' have been diligently created to assuage 'public concern'. National and international scientific organizations have repeatedly addressed the question of unique risks associated with genetically engineered organisms: rDNA-modified organisms are not inherently risky and rDNA-manipulated plants do not present any special risk of gene transfer compared to other genetically modified plants.

On the other hand, the challenges that confront the world's agriculture are expanding at an alarming rate. There is an immediate and critical need for new, environmentally safe pest-control strategies that are less input intensive. There is an immediate need for plants with greater drought and salt tolerance and for crops with enhanced nutrient content. There is therefore also an immediate need for small, entrepreneurial biotechnology companies that can translate advances made in academic and governmental laboratories into tangible products and technologies for farmers and consumers. Yet all of this is placed at risk, or, to be more precise, is retarded, by drains on R&D funding caused by unnecessary regulatory requirements on early field research. Max Planck said, more than half a century ago, that an important scientific innovation rarely makes its way by gradually winning over and converting its opponents; rather, its opponents gradually die out and the succeeding generation is familiarized with the idea from the beginning[9]. Time is thus on the side of biotechnology in the longer term. There is no doubt that, in a decade or two, many products made with the new biotechnology will be as much a part of our routines as microwave ovens and computers are to children today. However, that is not good enough. For many reasons, including those pertaining to economic equity and justice (the poor suffer most from unnecessarily high consumer prices) it would be tragic if its acceptance and potential growth were even minimally limited or delayed – for those at or below the subsistence level, justice delayed is justice denied. The greater the unnecessary burden and expense for research and development using biotechnology, the more likely that the earlier biotechnology products will be higher-value-added ones, available predominantly to wealthier countries and populations and at higher prices than necessary.

The battle for the adoption of sound public policies on biotechnology is not an intellectual exercise akin to a debate at the Oxford Union; rather, it is a real-life struggle for the availability of products that will enhance, prolong and enrich lives – and for the private sector's ability to develop and market such products without undue interference.

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Biopolymers from marine prokaryotes

Ronald M. Weiner

Biopolymers from marine prokaryotes, both Bacteria and Archaea, offer a number of novel material properties and commercial opportunities. The characteristics of marine exopolysaccharides and melanins that enhance the survival ability of the organisms producing them can be exploited for a number of products ranging from emulsifiers to adhesives. In the prokaryotes, the polyhydroxyalkanoates form carbon-storage molecules, but their technological application is entirely different, serving as a potential base material for biodegradable plastics. Marine biopolymers are a significant and undeveloped biological resource.

Prokaryotic biopolymers are an important resource and are finding increasing uses in the biotechnology and biopharmaceutical industries. Capsular, complex extracellular polymeric substances (EPS) alone have multiple industrial applications. For example, *Xanthomonas campestris*, a plant pathogen, synthesises xanthan gum (Fig. 1), which is found in a number of different foods and has numerous industrial applications[1]. Similarly, marine biopolymers represent a vast resource of untapped potential, for only a proportion of marine prokaryotes have been cultivated[2]. This article discusses three general classes of polymers that are synthesised by marine prokaryotes (Table 1), and describes their function in nature and their potential and actual commercial applications.

The structural, functional and utilitarian diversity of extracellular polymeric substances

EPS are usually composed primarily of high molecular weight capsular polysaccharides (CP) and have many important functions in nature, including protecting parasitic organisms from phagocytosis. Their major function in biofilm composition (Fig. 2) and physiology has recently gained much attention, as has the significance of these biofilms[3]. EPS can function variously as surface adhesins, in protection from predation and desiccation, in ionic interactions, as enzyme 'stabilisers', or concentrators, as storage or nutrient reserves and as dispersants (emulsans), to release bacteria from nutrient-spent surfaces[4,5].

Function follows structure, and the many roles EPS play in nature are a reflection of their considerable species-specific structural heterogeneity. They range from simple α-1-4-linked, unbranched glucose polymers (dextrans) to highly complex, branched and substituted heteropolysaccharides made up of repeating oligosaccharide subunits such as xanthan and colanic acid[6]. CP can also be substituted with pyruvate, acetate, formate, sulfate, phosphate and other groups. Two identical sugars can bond to form 11 different disaccharides, whereas two identical amino acids can form only one dipeptide. Additionally, CP contain a wide variety of sugars[7] and, in some cases, several non-carbohydrate side groups. This produces a heterogeneity that far exceeds that of proteins. CP can also be complexed or bonded to various fatty acids, proteins, hydrocarbons and other polymers of various sizes. Within a species, the length of EPS may be uniform or heterogenous, and their molecular weight ranges from 50 000 to over several million kDa[8].

The numerous existing and potential applications of EPS are reflections of their many proposed functions in nature[4] and their vast array of chemical compositions, bonding structures and architectures. For example, alginic acid (structurally, one of the least complex) is a repeating linear heteropolymer of mannuronic and guluronic acid (Fig. 1), with the exact molar ratios varying between species[1] and some domains of the polymer having alternating sugars while other domains are homopolymeric. This one type of CP alone has a wide range of applications, based on various properties including its ability to emulsify, retain water, form gels and control ice crystallization. As with other commercially available EPS, even these properties can be modified by subsequent treatments, including, in this case, various salts (ionic alteration) and propylene glycol (covalent alteration). Alginates are ingredients of hundreds of different types of processed food, and are also used in the paper,

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PII: S0167-7799(97)01099-8

adhesive, textile, ceramics, paint and chemical industries[1]. Nonmarine bacteria and algae also synthesize alginic acid, which is currently mainly obtained through seaweed harvesting, but it is possible that marine bacteria that synthesize these polymers will soon be exploited leading to more reliable and potentially less expensive product.

Rosenberg and co-workers have worked with several EPS with a number of interesting properties. One group of EPS, isolated from bacteria that colonize fish, has drag-reduction properties; it is speculated that, in nature, these polymers enhance fish mobility, while the surviving fish provide a nutrient-rich substratum[9]. Another type of EPS are emulsans[10,11]; in nature, emulsans, synthesized most notably by *Acinetobacter*, are believed to desorb oil-degrading bacteria from 'spent' hydrocarbon droplets[12]. Applications for both of these polymers by industries including petroleum and shipping[12] are shown in Table 1.

An array of promising applications makes use of the ability of many marine bacteria to adhere to various substrates. Marine prokaryotes can attach under pressure, in brine and at temperatures of 4°C or less, and the binding is sufficiently strong to overcome considerable currents[3]. The adhesive (bonding of dissimilar molecules) properties of some EPS make them promising marine cements[13] and their cohesive (bonding of like molecules) properties, necessary for biofilm accretion, suggest they possess a potential function in coatings. They are also relatively nonimmunogenic[5], enhancing their potential as bioadhesives[14].

Furthermore, many of the EPS are anionic and can bioconcentrate cations more than 10 000 times[15]. An important mechanism for metal scavenging by EPS is the formation of salt bridges between metal ions and the carboxyl groups on acidic polysaccharides, in which polyvalent metal ions bind to two anionic groups on two separate polymer chains. EPS also absorb a variety of organic compounds. These properties make them particularly useful for the bioremedation of heavy metals and metal-containing pollutants[16]. Only a small proportion of the existing EPS have yet been discovered[2] and, of these, an even smaller proportion have been characterized.

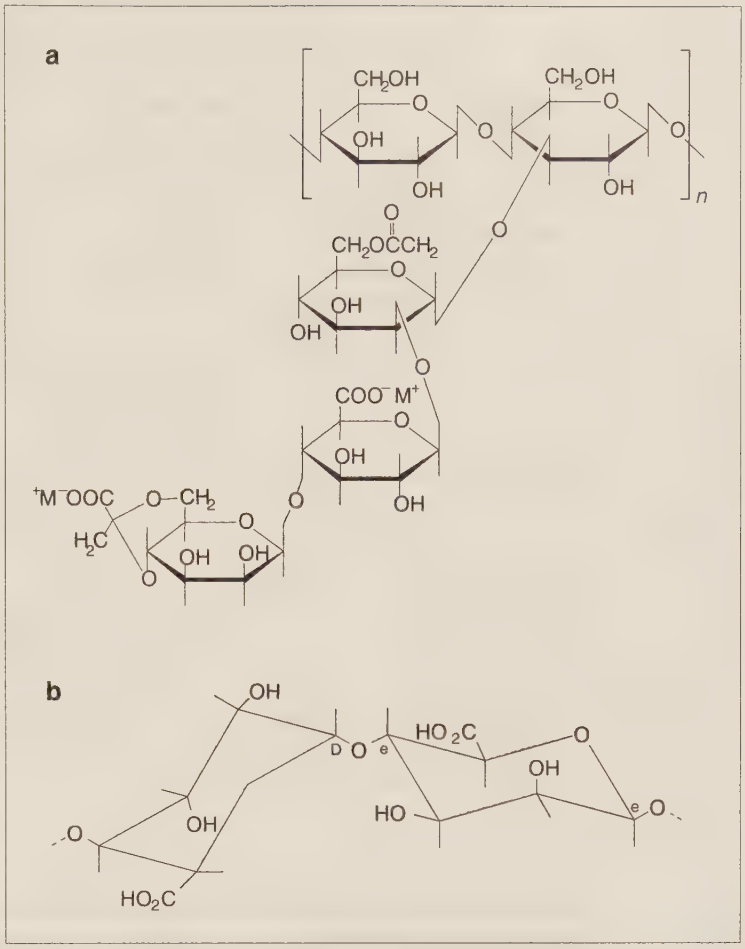


Figure 1
Structures of two industrially important capsular extracellular polymeric substances. (**a**) Xanthan gum, a branched, complex, substituted heteropolymer. (**b**) Guluronic-acid–mannuronic-acid heteropolymer domain of alginic acid, a linear molecule.

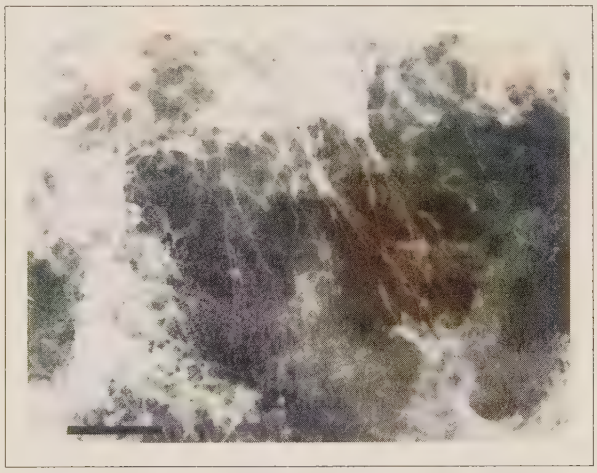


Figure 2
Scanning electron micrograph of a biofilm of a pure culture of *Hyphomonas*, showing cells attached to the substratum by strands of extracellular polymeric substances. Bar = 10 μm. Courtesy of S. Langille.

Bacterial melanins

While EPS serve as a general example, two additional classes of marine biopolymers are equally promising undeveloped resources. Many of the strains of marine bacteria that have been cultivated express a number of pigments. As with certain EPS, some pigments are also produced by eukaryotic organisms but, in many cases, prokaryotes offer greater potential for commercialization because of the promise of cheaper fermentations and purification. One such class of pigments are the melanins. In nature, although melanins are not essential for growth, they increase the survival capability of the organism in many environments. They protect DNA and other molecules from ultraviolet light, enhance virulence, inhibit biofilm degradation, protect enzymes from protease activity, protect microorganisms from hydrolytic enzymes and may even act as proton and nutrient sinks in biofilms[17].

There is considerable heterogeneity among the melanins, although, unlike EPS, they can be classified

Table 1. Biopolymers from marine bacteria with current or potential commercial application

Types	Polymer	Potential application(s)	Refs
Complex polysaccharides and related extracellular polymeric substances (EPS; normally capsular)	Adhesins[a]	Underwater surface coatings, bioadhesives	5,13
	Drag reducers	Drilling, ship efficiency	9,12
	Emulsan	Oil cleaning and viscosity reduction	10–12
	Surfactant	Dispersing agent, grinding aid	12
	Alginate	Food, textile, etc.	1
	Metal-binding EPS	Toxic-metal bioremediation	16
Pigments	Melanins	Biotechnology, reporter gene, cosmetics, dyes, colorings, sun screens	17–20
Polyesters	Poly 3-hydroxyalkanoates (e.g. polyhydroxybutyrate)	Biodegradable plastics	23,25,28

[a]Defined as prokaryotic extracellular polymeric substances (EPS) that enable prokaryotes to adhere to surfaces, causing biofouling, and that may be involved in biofilm accretion.

into several groups based on their synthetic precursors (Table 2). Also unlike EPS, the pigmentation phenotype is rather easily cloned, because generally only a single enzyme is required for melanisation[18]. For example, eumelanins originate from the action of tyrosinase[19], while pyomelanins originate from para-hydroxyphylpyruvate-hydroxylase activity[20]. Although melanins are complex, high molecular weight compounds, their synthesis results from auto-oxidative reactions from active precursors. As such, they are strong reducing and free-radical-capturing agents.

Other applications of melanins make use of their chromophoric properties (Table 1). For example, the gene coding for parahydroxyphenylpyruvate hydroxylase, which catalyzes the synthesis of the pyomelanin precursor homogentisic acid (Fig. 3), may be an excellent reporter gene (indicator of gene expression) because: (1) it has a sequence that is relatively conserved[21] and does not form obvious secondary structure[18], and should therefore be efficiently expressed in most species; (2) the end products of its action (homogentisic acid, pyomelanin precursors and pyomelanin) are not toxic; (3) only a single gene need be inserted; (4) the entire sequence is known for the gene from the marine bacterium *Shewanella colwelliana*[18] (shown in batch culture in Fig. 4), leading to easy manipulation; and (5) pyomelanins are obvious, dark red, water-soluble indicators that require no

Table 2. Common types of melanins

Melanin[a]	Precursors[b]	Pigmentation
Eumelanin	Dihydroxyphenylalanine (DOPA)	Black
Phaomelanins	DOPA quinone and glutathione or cysteine	Brown, red or yellow
Allomelanins	Catechol	Brown
Pyomelanins (alkaptons)	Homogentisic acid	Brown or red

[a]General group of complex polyphenolic heteropolymers. Other (less well documented) classes of melanin also exist[17].
[b]There are a variety of pathways by which tyrosine can be converted to melanin.

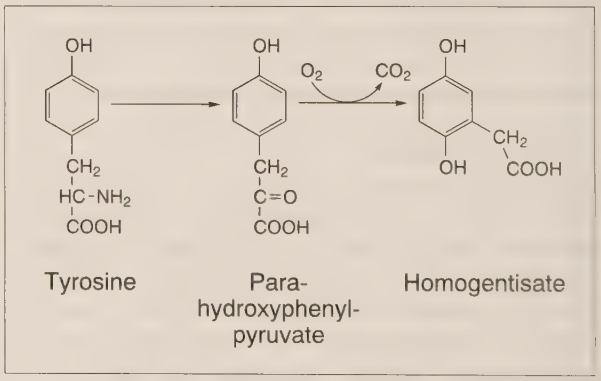


Figure 3
The precursor to pyomelanin (a low molecular weight melanin) is homogentisate, which is synthesised from *p*-hydroxyphenylpyruvate by the action of *p*-hydroxyphenylpyruvate hydroxylase (EC 1.13.11.27). *p*-Hydroxyphenylpyruvate is synthesised from tyrosine as part of the tyrosine degradative pathway. When homogentisate is in excess, it can undergo oxidation to form pyomelanin (structure unknown).

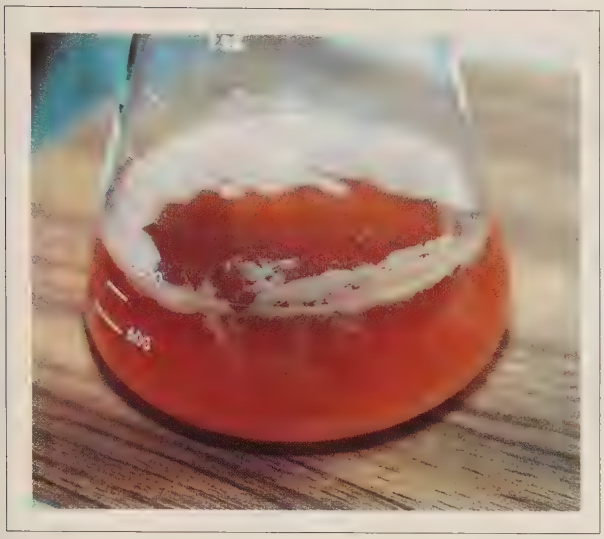


Figure 4
Batch growth of the marine bacterium *Shewanella colwelliana* in early stationary phase. The brownish-red pigment is pyomelanin and the fluffy white material is the extracellular polymeric substance.

developer. Melanins have a range of chromophoric properties that can also be exploited for sunscreens, dyes and coloring[22]. Other promising applications take advantage of the fact that melanins sequester different kinds of organic compounds, including fungicides and antibiotics, which may allow them to act as slow-release agents[17].

Polyhydroxalkanoates impart environmental plasticity to prokaryotes and environmental compatibility to plastics

The final example of marine prokaryotic polymers that have commercial promise are the poly-β-hydroxyalkanoates (PHAs), a class of aliphatic polyesters. This subject has recently been reviewed[23] and, as an important polymer, it is summarized here, emphasising the ecological aspects. In marine prokaryotes, PHAs accumulate intracellularly in large amounts (ranging from 25–80% cellular dry weight) when the organism is in a high-nutrient econiche such as a biofilm, particularly one with a high carbon:nitrogen ratio. As a food reserve, PHAs are metabolised for carbon and energy by prokarotes when they are in low-nutrient econiches such as the mid-ocean. Unlike the earlier examples, complex polysaccharides and pigments, PHAs would be used industrially for a totally different function than they serve in nature; they could be used to form biodegradable, thermoplastic polymers, whereas, in nature, polyesters function as a prokaryotic carbon-reserve material.

PHAs have the general structure shown in Fig. 5. The lowest molecular weight PHA, poly-β-hydroxybutyrate (PHB), is the most common in nature. However, their molecular weights can be up to two million, which corresponds to approximately 20 000 monomers per polymer molecule. Many prokaryotes, for example halobacteria[25] and hyphomicrobia[26],

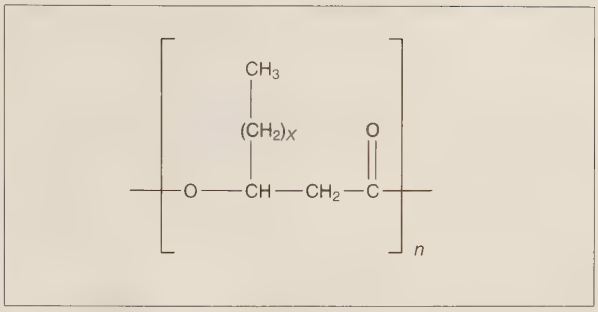


Figure 5
General structure of poly-β-hydroxyalkanoates (PHAs). In poly-β-hydroxybutyrate (PHB), x = 0.

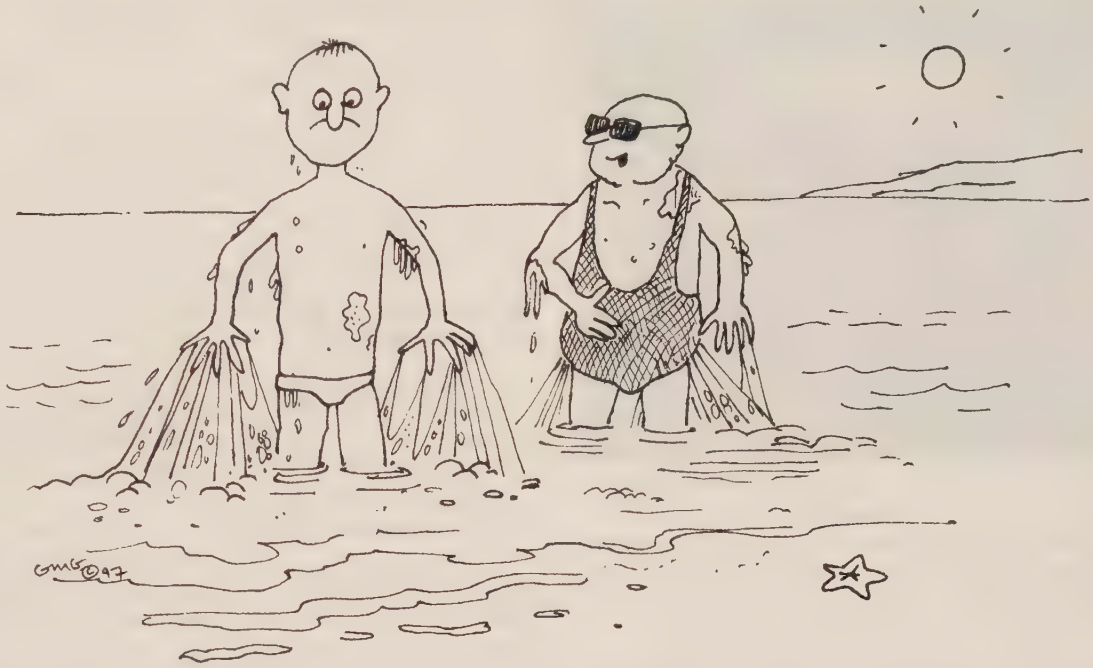
synthesise PHAs and, when carbon and energy are required, the PHA is normally depolymerized to D(–) hydroxybutyric acid and then metabolised to acetoacetate and acetoacetyl-CoA.

The usefulness of the prokaryotic PHAs comes from their thermoplasticity, a property found in numerous plastics including polystyrene, polycarbonate and polypropylene. The significant difference is that prokaryotic PHAs form the base material of biodegradable plastics[25]; for example, PHB has a similar structure to polypropylene, which is used in over 200 products, including pipes, packaging materials and domestic appliances. Biodegradability is enhanced because, unlike (synthetic) polypropylene, (prokaryotic) PHB does not float, but sinks to the sediment, where it can be degraded in approximately one month, rather than the year it would have taken had it remained on the surface, all other conditions being equal[27].

The properties of PHAs, like those of the complex polysaccharides and pigments described above, can be altered and enhanced by manipulating or modifying their chemical composition. For example, homopolymers of PHB are brittle, whereas copolymers of PHB and 3-hydroxyvalerate have lower melting temperatures, resulting in increased flexibility and resiliency[28]. Copolymers of various proportions of each building block can be engineered by manipulating the carbon sources supplied during growth[25].

Until an economical high-yielding marine-growth medium is formulated, the development of genetic-engineering methods for PHA synthesis is the most promising method of production. In *Alcaligenes eutrophus*, for example, there are three genes for the synthesis of PHB that have been successfully cloned[29]. The cloning vehicle can be chosen to produce high yields in existing growth media. In either case, for the foreseeable future, PHA will be produced by prokaryotic fermentations[23], using either natural or engineered organisms. It might be quite some time, if ever, before transgenic plant technology is possible and cost effective.

While prokaryotic polymers remain more costly to produce than those from other sources, the key to their future (their minimal impact on environmental deterioration) lies in two facts – they are biorenewable and many are biodegradable and/or nontoxic. For example, in 1982, ICI began to market PHA-derived



"and the marine biopolymers are particularly good this year!"

plastics under the trade name Biopol. While Biopol is still more than twice as costly to produce as other plastics, it remains commercially important because it is biodegradable and so, when environmental remediation is also considered in the productivity equation, Biopol becomes cost competitive in an environmentally conscious society.

Isolations of novel marine prokaryotes are becoming commonplace. There have been innovative advances in fermentation technology and analytical chemistry, leading to better product characterisations. With these developments, many more marine prokaryotic biopolymers will find their way to the marketplace.

Acknowledgments

Special thanks go to R. Colwell and E. Rosenberg, both of whom made substantial contributions to the development of marine biotechnology, and provided valuable insights to this paper. A grant from the Maryland Industrial Partnerships and Oceanix Corp. supported RW.

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Protein-monolayer engineering: principles and application to biocatalysis

Claudio Nicolini

Highly ordered protein monolayers with defined structures and functions can be generated using appropriate modifications of the original Langmuir–Blodgett method. The characteristics of such monolayers of proteins, and enzymes in particular, are summarized, together with an evaluation of their potential applications to biocatalysis. None of the other immobilization techniques confers such long-term protection and thermostability on the structure and function of immobilized proteins.

Monolayer engineering aims to create complex molecular assemblies with a specific layered structure. The techniques used are based mainly on the original Langmuir–Blodgett (LB)[1] or Langmuir–Schaefer (LS)[2] methods, often combined with self-assembly[3] and adsorption processes. Typically, monolayers of amphiphilic organic molecules are formed at the liquid–gas interface in a LB trough by first spreading and then compressing the organic surface layer to a defined surface pressure. The monolayer thus formed is transferred onto a solid substrate[1,2] that is arranged vertically and dipped through the monolayer. Successive layers are built up by repeating this process. However, protein monolayers are usually prepared by adopting the horizontal-lift LS technique[4,5] (modified to avoid the protein denaturation that would otherwise result from the high surface tension existing at the air–water interface) for transfer onto the solid substrates. For enzymes, an adsorption trough[6] has proved to be even more effective in preserving the native enzyme function in the engineered monolayers[7]. More than 15 proteins, both water-soluble and membrane-bound, have been investigated[3–7] and extensively characterized by a wide variety of biophysical techniques[4,5]. Promising applications of such technology to nanoelectronics are described elsewhere[5,8].

How protein monolayers are formed

To produce a film by classical LB or LS techniques[1,2,9,10], a solution of amphiphilic molecules (i.e. molecules consisting of hydrophilic groups that interact strongly with water and hydrophobic hydrocarbon chains that do not) is spread at the air–water interface (Box 1). As proteins are not ideal amphiphilic molecules, the techniques need to be adapted, either by chemical methods (for example, using reverse micelles[11], derivatization techniques[12] or varying the subphase composition[13,14]) or by protein engineering[4,5,12], in order to preserve their native structure and function in the monolayers[3,11–22]. The horizontal-lift LS technique is better suited to proteins and provides a homogeneous and reproducible monolayer coverage of the substrate[23–29]. Various modifications to this approach were introduced by Kuhn[24,25].

The quality of protein-monolayer formation at the air–water interface (critical in the case of enzymes) is related to the degree of preservation of the native properties of all proteins. The magnitude of the electrostatic forces maintaining the protein structure is comparable with that of the surface tension and so, during the formation of protein monolayers over the water surface, there is always a risk of protein denaturation. There are some examples (e.g. cytochrome c) where such behaviour occurs readily but can be overcome by utilizing reverse micelles[11] or protein derivatization[12]. A new, alternative, approach that we implemented for engineering enzyme monolayers[6], but which should be suitable for most proteins, is to dip a plate, closely adjacent to the substrate, through the monolayer: when the substrate is withdrawn, together with the plate (Fig. 1), a layer of water is held between the plate and the substrate by capillary forces[6], preventing any denaturation of the adsorbed protein monolayer.

Only for transmembrane proteins, such as photosynthetic reaction centres (RC; from *Rhodobacter sphaeroides* or *Rhodopseudomonas viridis*) and bacteriorhodopsin (in the purple-membrane fragments of

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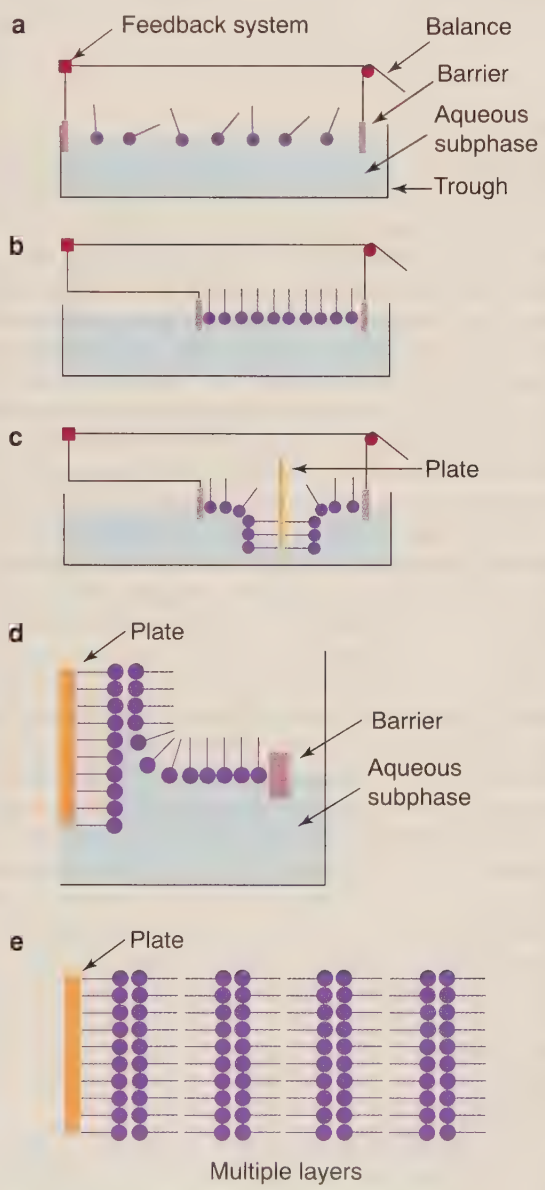
PII: S0167-7799(97)01084-6

Box 1. The deposition process for thin films

In order for the molecules to spread, they must be dissolved in a 'strong' solvent at a concentration that does not permit the formation of aggregates. As shown in the figure below, drops of the solution are initially placed on the water surface (**a**). The number of molecules in the drop must be small enough to form a monolayer in which the molecules are far enough apart to prevent interaction after spreading. When an amphiphilic molecule is placed on the water surface, its polar head group interacts with the water, while the hydrocarbon chain does not, and is oriented away from the water and towards the air. Floating molecules can be compressed by the barrier until a condensed state is achieved (**b**); the resulting monolayer is transferred on the substrate by a vertical lift (classical LB) technique (**c**). By reiterating the procedure, successive multilayers can be deposited (**d–e**).



Halobacterium halobium), is the protein structure not affected by spreading over the water surface. For RC, the spreading protein solution consists of individual protein molecules surrounded by surfactant molecules; after spreading, there are both protein and surfactant molecules at the air–water interface. As these surfactant molecules have short hydrocarbon chains (12 carbon atoms) that prevent them from reaching high surface pressures, they can be removed from the surface by applying several compression–expansion cycles. For bacteriorhodopsin, the spreading protein solution consists of membrane fragments. Deposition is difficult, as the fragments are mainly hydrophilic with only small hydrophobic moieties; most of the protein molecules penetrate the volume of the aqueous subphase instead of participating in monolayer formation, although this can be prevented by using a concentrated salt solution as the subphase. This has the side-effect that, because the salt is transferred with the film onto the substrate, the sample must be washed after the deposition of each layer.

All proteins, including enzymes, whether membrane-bound or water-soluble, retain their structural properties after deposition onto solid substrates by the 'modified' LB method[4,5,11–14], as confirmed by various techniques (Box 2 and Fig. 2). The surface concentration of proteins, which can be estimated indirectly from compression-isotherm analysis, can be measured directly on solid substrates by techniques such as nanobalance and atomic-resolution microscopy[5]. Where enzyme monolayers are engineered for biocatalytic applications, either the 'modified' LS or the adsorption-trough technique may be used; while the latter preserves the functionality of the native enzyme in solution with some degree of thermostability (over 80°C for a limited time), the former generates a uniquely thermostable enzyme with an apparently intact native structure, but of limited activity in solution.

Engineering thermostable and long-lived films

A common feature of all protein LS films is the high thermal stability of the protein structure and function[3–8,11–22]. The first question we addressed concerned the preservation of the protein secondary structure in LS films under the thermal treatment originally reported for transmembrane proteins[4,30]. The thermostability of protein structure has been firmly established for LB films of proteins of various classes, including RC[4], glutathione-S-transferase (GST)[16], alkaline phosphatase (AP)[13], bacterial[22] and bovine[19] rhodopsins, urease[19], thioredoxins[29], cytochrome P450[8] and azurin metalloproteins[29] (Box 3). Nearly all retained their secondary structure up to 200°C when incorporated into films (Box 3). The circular-dichroism data confirm that, in all these cases, protein secondary structure is unaffected by raised temperature (Fig. 2, Box 3); at the same time, activity measurements of protein monolayers exposed to increasing temperatures (Fig. 3) confirm that even the enzyme function is thermostable in LS films (Box 3). The enzymatic activity, even though dramatically reduced by the LS

procedure[7], also appears to be 'heat-proof' in LS monolayers. In contrast, when the enzyme is in solution, this property is completely lost. Interestingly, the enzyme activity is at maximum when the deposition is onto a chemically activated surface[16].

The enzymatic activity of thermally treated GST, immobilized as a monolayer in LS films and stored for one year at 4°C, was determined at room temperature[16]. Stored enzyme films remained active and thermostable, indicating that the silanizing agent used for the covalent binding of enzyme films to solid supports does not affect the properties of the enzyme in the film. Similarly, LS films of bovine rhodopsin (maintained in silica gel, in the dark and at room temperature for over one year) were also stable. In summary, all the enzymes immobilized in films possessed greater stability than when in solution. The functional activity and the secondary structure of the proteins remain unaltered after heating to 150°C (Figs 2 and 3) and raising the surface pressure to 45 mN m^{-1} (data not shown). The secondary-structure analysis also revealed a higher thermal stability in films. Circular-dichroism spectra of AP in film and in solution are similar at room temperature; the minor difference between the two spectra may be attributed to the orientation of molecules – in film, it is assumed that they may have some preferred direction, whereas the orientation in solution is random. However, the spectra respond quite differently upon heating – the spectrum of film-immobilized enzyme remains almost unaffected up to 200°C, while in solution, the spectra has changed dramatically by 100°C (Fig. 2). The preservation of structure and functional activity has been observed for protein films up to 200°C, probably as a result of the close molecular packing and two-dimensional order[7,8,14].

Finally, when performing its catalytic function, an enzyme molecule is subject to reversible conformational changes that could be limited by the high degree of packing in a film. When AP from calf intestine is bound to Hybond-N+™ membranes, either by direct spreading or by LS film deposition, direct monitoring of product formation by spectrophotometry reveals that:

(1) no detachment of the enzyme occurs;

(2) there is a consistent loss of activity for the spread films, but not for the LS-deposited enzyme, after drying of the membrane at room temperature in a desiccator – stable 'heat-proof' activity is found in the LS monolayer but not in the spread; the salting-out of the LS protein monolayer from aqueous solutions is achieved with a high ionic force, yielding a high surface pressure (up to 60 mN m^{-1}) with 3M NaCl;

(3) a more significant loss of activity occurs during spreading of the film than after LS deposition and drying at room temperature[13].

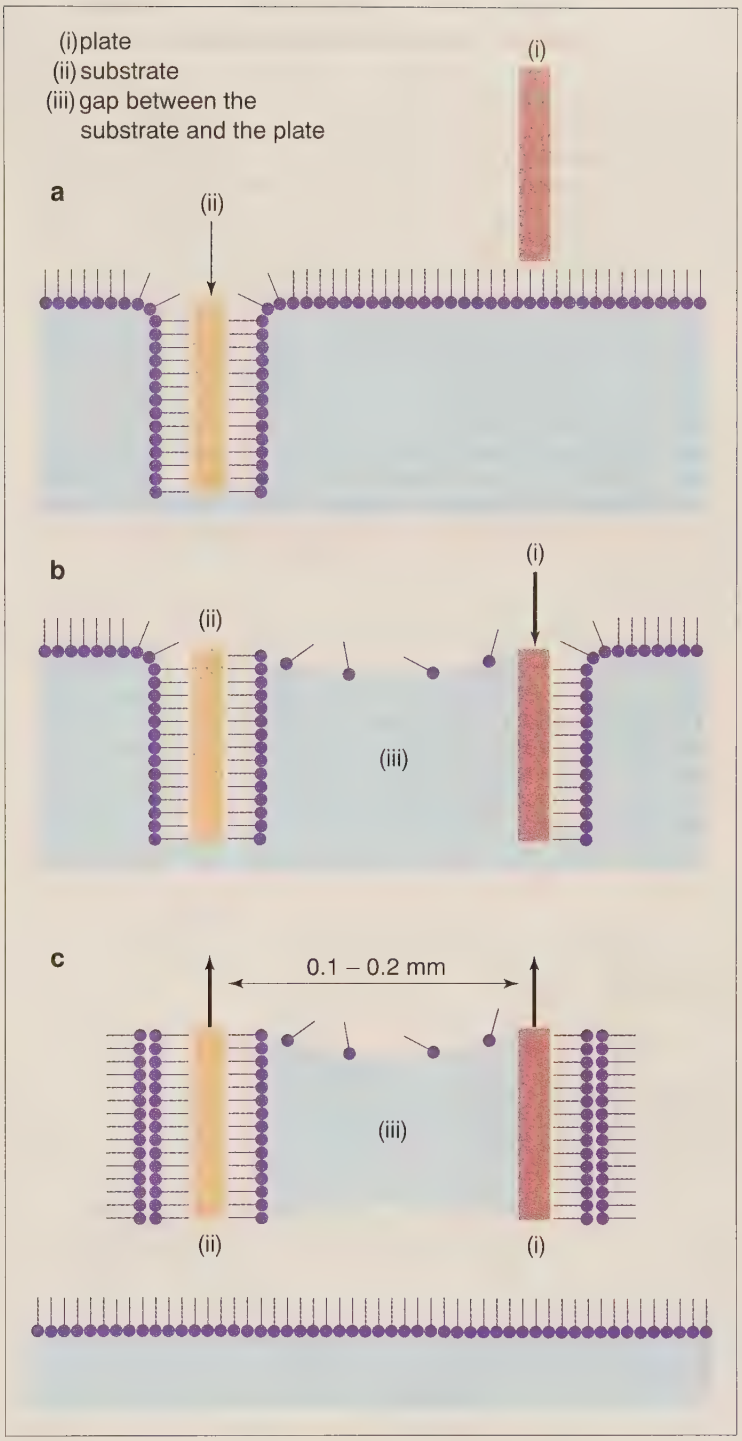


Figure 1

The ELBA adsorption trough, which uses a 'protecting-plate' method for the deposition of films. The monolayer is deposited on the first plate by dipping it vertically into the aqueous phase (**a**); the second plate is then inserted close to the first, capturing water between the two plates (**b**); the plates are finally pulled out from aqueous phase, capturing some water between the two plates by capillary action (**c**). Thus, the monolayer is protected by the layer of water, producing a film with its hydrophilic surface outermost.

Monolayer engineering compared with other immobilization techniques

Protein-based catalysts are less stable than many traditional chemical catalysts, particularly in water-miscible organic solvents. These commonly used solvents disrupt the tertiary structure of the enzyme, so inactivating it. The industrial use of enzymes is, in many instances, limited by their lack of stability and robustness, variable activity and high cost. Commodity enzymes, where biocatalyst homogeneity and purity

Box 2. Characterization techniques

Protein-monolayer films can be characterized by almost all known biophysical techniques – circular dichroism, atomic-force microscopy, scanning–tunnelling microscopy, transmission electron microscopy, differential scanning calorimetry, nuclear-magnetic resonance, infrared and electron-paramagnetic-resonance spectroscopy, X-ray diffractometry, absorption spectra, nanogravimetry, ellipsometry, CCD high-resolution image analysis and electric (DC and AC) measurements.

However, measuring the surface potential is a method unique to the characterization of LB films. There are two main methods for measuring the surface potential; in both, one electrode is embedded into the water subphase while the second is placed above it. The more common method is based on the Kelvin probe[34], in which the upper electrode vibrates. Oscillations of the electrode modulate the capacitance of the gap between the electrode and the water surface. As a result, alternating current appears in the circuit connecting the vibrating electrode and the water subphase. The value of the current depends on the amplitude of the oscillations and upon the value of surface potential, and so the surface potential can be measured by fixing the amplitude.

Brewster-angle microscopy is a very useful tool for the visualization of the domain structure of the monolayer; this reveals the domain structure without the need for any additives in the monolayer, and therefore without affecting its structure. Brewster microscopy is based on the fact that light incident on the surface at the Brewster angle is reflected and partially polarized. The value of the Brewster angle depends upon the material at the surface, and so it is possible to visualize the domain structure of the monolayer, as the presence of the monolayer changes the Brewster angle.

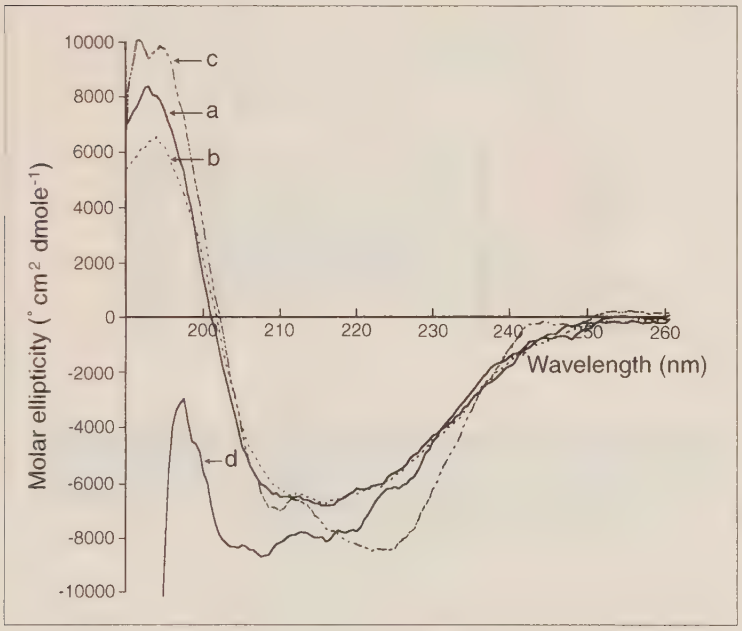


Figure 2

Structural preservation of proteins in thin films. The graph shows the molar ellipticities of alkaline phosphatase in film (20 layers) at 25°C (**a**) and 200°C (**b**) and in solution (0.025 mg ml^{-1}, optical path 2 mm) at 25°C (**c**) and 100°C (**d**).

are relatively less important, have been developed primarily for detergent and food applications. To increase the use of biocatalysts in the fine-chemical and pharmaceutical industries, several immobilization techniques have been introduced, based on adsorption or anchoring of the biocatalyst to inert matrices such as cellulose microfibres, glutaraldehyde-fixed polymeric reticules or functionalized ossiranic resins. Immobilization of the pure enzyme allows the recovery and reuse of the biocatalyst in large-scale chemical-production systems, but the inert support results in a significant dilution of functional activity, increases cost and does not improve stability. Recently, a new technique – cross-linked enzyme crystals (CLEC)[31] – was introduced that overcomes these obstacles by crystallizing the protein catalyst and then cross-linking the pure microcrystals with glutaraldehyde. Such enzymes remain active for multiple cycles, in near-anhydrous organic solvents and at temperatures of up to 50°C. However, several limiting features remain that are absent in monolayer engineering, namely the need to use crystallizable proteins of high purity and the poor accessibility of the substrate to the protein. No other immobilization technique, including self-assembly, membranes and anchoring to lipid monolayers, results in a comparable thermostability or long-term stability of the native protein structure and function[9,10,32,35–39].

Box 3. Thermal stability and long-term preservation

One common feature of all protein films is the high thermal stability of the protein structure (secondary and higher orders) and function, as shown in Table 1; almost all the proteins investigated demonstrated the preservation of secondary structure up to 200°C when organized into a LS film. Similarly, the functional activity of a wide variety of membrane-bound and water-soluble proteins appears to be preserved up to 150–200°C in LS films.

Considering that most of the proteins studied are water soluble and all stem from mesophilic organisms, this heat stability is a very significant result that may have far-reaching implications. The long-term functional preservation at room temperature of LS films also turned out to be an important general property. The whole sequence of structural alterations induced in protein LB film with increasing temperature indicates that, first, a progressive loss of dehydration occurs, followed by loss of functional activity and tertiary–quaternary structure, and only at quite high temperatures is the secondary structure lost.

Biocatalysis based on enzyme monolayers

Enzyme films may be immobilized onto small spheres (Fig. 4), thereby increasing the number of enzyme molecules exposed to the medium and increasing reactor productivity. The increased surface-to-volume ratio of spheres compared to flat supports increases the number of enzyme molecules in a given volume of a reactor. As most enzymatic reactions proceed in the liquid phase, enzyme molecules must be chemically attached to the surface of the spheres to prevent detachment[16]. A technique suitable for the deposition of enzyme LS films onto glass spheres has been developed for enzyme-catalysed processes: the sphere surface is activated by a siloxane polymer, and then the activated spheres are distributed onto a film

Table 1. Functional and structural 'melting' temperatures (°C) of proteins in solution and in LB films

	Glutatathione-S-transferase	Alkaline phosphatase	Thioredoxin	Cytochrome P450scc	IgG	Rhodopsin	Photosynthetic reaction centres
Solution secondary and higher-order structure	60°C	60°C	60°C	~60°C	60°C	75°C	55°C
LB secondary structure	150°C	–	190°C	>100°C	200°C	200°C	~200°C
LB tertiary–quaternary structure	–	–	–	–	–	–	110°C
Solution functional activity	60°C	60°C	–	–	–	~75°C	~60°C
LB functional activity	>150°C	150°C	–	–	190°C	~200°C	110°C
Solution dehydration	–	–	–	–	–	–	50°C
LB dehydration	–	–	–	–	–	–	100°C

–, not determined.

of enzyme (urease or GST) at the air–water interface. The spheres, with a bound enzyme layer, are finally collected and dried[22]. Spectrophotometric measurements revealed that the functional activity of these enzyme films was preserved even after heating to 150°C. Such enhanced thermal stability increases the potential of protein films. Indeed, yields from reactors based on enzymatic catalysis might be improved by increasing the temperature of the reaction and using enzymes deposited by this technique.

A significant proportion of the functional activity of all the enzymes studied so far appears to be preserved upon immobilization in LS films, even though the degree differs from case to case[7]. Brewster-microscopy measurements (Box 2) give real-time evidence that enzymes only form stable LS monolayers up to a critical surface pressure, above which the layers collapse. LS films of GST, urease and AP deposited over 30 minutes on nonsilanized silicon substrates have activity values that are in the range 2–9 % of the solution values. GST is a member of a family of enzymes that play a physiological role in the detoxification of potential alkylating agents, including pharmacologically active compounds. The enzymatic activity of GST films was analysed spectrophotometrically, following the reaction kinetics of glutathione with 1-chloro-2,4-dinitrobenzene, which gives the product 1-glutathione-2,4-dinitrobenzene. The kinetic constants of the Michaelis–Menten equation, k_m and V_{max}, were estimated and compared to the solution values[15,16]. The samples were heated to 150°C, but still exhibited significant catalytic activity. The association constant, k_m, was close to the value in solution, but the maximum rate, V_{max}, was significantly lower. The activity and thermal stability remained at the same level following long-term storage for over one year.

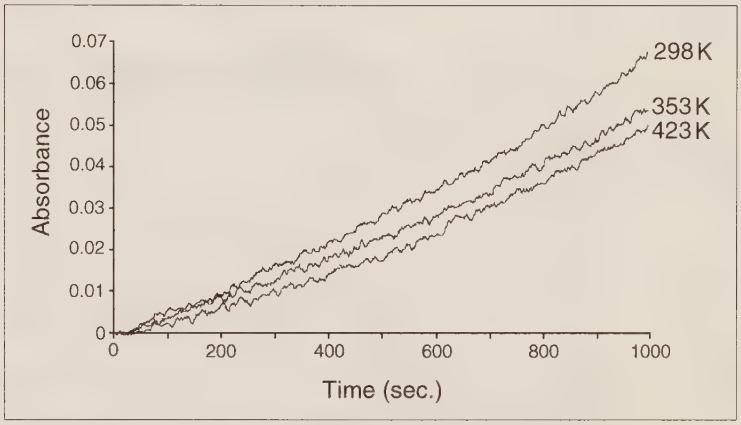


Figure 3

Graph showing the activity of urease after dry thermal treatment in an LB film deposited on glass spheres at three different temperatures: 298 K (room temperature), 353 K and 423 K. The measurements of enzyme activity were carried out at room temperature.

Outlook

The understanding of the thermal stability of proteins and its improvement are very important goals in both fundamental and applied science. This requires considerable knowledge of all the parameters involved in the structural stabilization of protein structure and the ability to control them for use as functional elements of biodevices and bioreactors. The possibility of modifying the thermal stability of proteins may increase their technological applications at high temperatures, allowing increases in the viscosity of the medium and enhanced reaction rates, which would lead to a reduction in the production of contaminants.

Recently, classes of proteins involved in processes such as electron transport, molecular recognition and biotransformations have become increasingly used for a variety of technological applications, ranging from biosensors and biotransistors to biocatalysis and bioactuators. Several of these applications require operating conditions with temperatures greater than 100°C, a condition under which most proteins denature, including a number of proteins isolated from extreme thermophiles.

Protein engineering, which may overcome these possible drawbacks, demands a specific procedure for

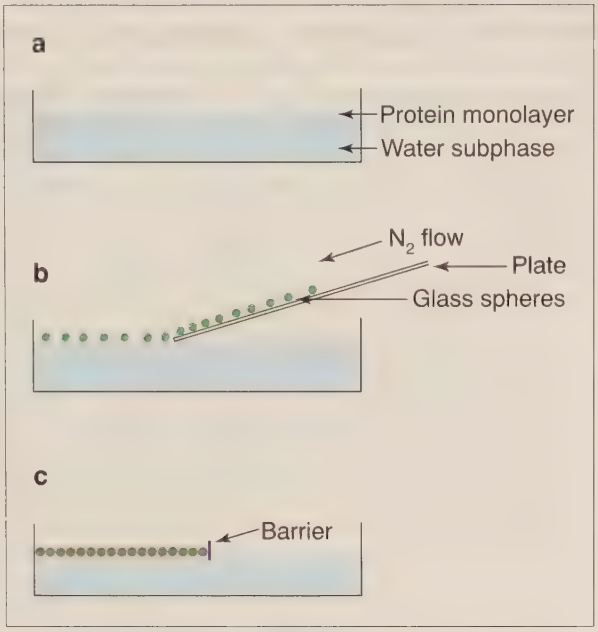


Figure 4

Summary diagram of the procedure for depositing protein films onto glass spheres. The protein solution is spread over the water subphase and the protein monolayer compressed to a surface pressure of 25 mN m^{-2} (**a**). Activated spheres are then distributed over the monolayer as follows: a plate carrying the spheres is moved along the monolayer, while a weak nitrogen flow is used to transfer the spheres from the plate to the liquid surface (**b**). The spheres are kept on the surface for 30 minutes in order to provide chemical linking of the monolayer to the activated surface. After this time, the plate is removed from the water. The monolayer and the spheres are compressed to the minimum area (just above that at which the monolayer would collapse) (**c**). The spheres are then collected, washed with substrate buffer to remove protein not chemically attached to the sphere surface, and dried.

each case and thus requires a considerable amount of research effort; the formation of a highly packed, two-dimensionally ordered protein film by the LB or LS technique could, however, be a general procedure for stabilizing protein structures at temperatures of up to 200°C (Ref. 3), whereas a protein normally denatures at approximately 55°C in solution. This has been shown to be effective for both water-soluble (e.g. antibodies, cytochromes) and membrane-bound (e.g. bovine and bacterial rhodopsins, RC from *Rhodobacter sphaeroides*) proteins. These conclusions were confirmed by X-ray studies with synchrotron radiation for dry self-assembled films of bacteriorhodopsin by Shen *et al.*[30] and, more recently, for dried photosynthetic-membrane films by Miyake *et al.*[33] It is also worth noting that the thermal behaviour of dry self-assembled smears of membrane-bound and water-soluble proteins is intermediate between the properties of the same proteins in solution and in LB films[3,4,14,22].

Biotechnological applications of 'heat-proof' enzymes can already be envisaged in a number of processes and more will emerge. The technology behind the formation of thin protein films based on the LB assisted-adsorption processes described above is effective in the preservation of enzymatic activity, but significantly less effective in terms of thermal stability when compared with the classical LB technique modified as described earlier. In order to explain this, a systematic comparative study was recently carried out on the same protein, isolated from both a mesophilic and a thermophilic microorganism, in both solution and in LS film. Proteins isolated from thermophilic microorganisms are often structurally similar to their mesophilic analogues, but dissimilar in their stability to heat, organic solvents, detergents, common protein-denaturing agents and proteolytic enzymes. Consequently, comparative studies might give further insights into the molecular mechanisms controlling protein thermostability[40].

Acknowledgements

I would like to thank Victor Erokhin, Sergio Paddeu and Vladimir Troitsky for their comments in the preparation of this work. The work has been supported by the Ministry of Universities and Technological and Scientific Research of Italy (Chapter 2102), by the ELBA Foundation and by the Polo Nazionale Bioelettronica-Scientific and Technological Park of Elba Island.

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Polyunsaturated fatty acids, part 1: Occurrence, biological activities and applications

Iqbal Gill and Rao Valivety

Polyunsaturated fatty acids form a unique class of food constituents that show a wide range of functions in biological systems. Investigations over the past two decades have uncovered their roles and those of their eicosanoid metabolites, and have highlighted their homeostatic functions in mammals. A growing number of common human medical conditions are thought to be traceable to dysfunctions in the eicosanoid system, which could in turn be due to imbalances in the intake and/or metabolism of polyunsaturated fatty acids. This, together with medical advances, has spurred the introduction of biomedical products, nutritionals, fortified foods and health supplements.

Polyunsaturated fatty acids (PUFAs; long-chain fatty acids containing two or more double bonds) have entered the biomedical and nutraceutical (food and nutritional products ascribed with specific health-promoting biological activities) arenas as evidence has mounted rapidly mounted over the past decade on their biological roles, especially in relation to certain disease conditions[1–6]. In addition to their potential pharmaceutical applications, the occurrence of PUFAs in foodstuffs, coupled with a heightening public awareness of 'healthy' foods, has brought these compounds firmly to the attention of consumers. In particular, $C_{18–22}$ PUFAs (see Glossary) containing three or more nonconjugated *cis* double bonds have come under intense scrutiny as their *in vivo* roles and potential applications in therapeutics and nutraceuticals have become clearer[2–6]. This article will focus on ω-6 and ω-3 PUFAs (see Glossary), exemplified by the essential acids linoleate (LA) and α-linolenate (ALA) and on the acids derived therefrom. Examples of higher ω-6 acids are γ-linolenate (GLA), dihomo-ω-linolenate (DHGLA) and arachidonate (AA), and common ω-3 acids include eicosapentaenoate (EPA), docosapentaenoate (DPA) and docosahexaenoate (DHA)[1,2]. Figure 1 shows the structures of all these PUFAs.

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Occurrence and production

PUFAs occur throughout the animal and plant kingdoms, although the richest diversity is encountered in

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PII: S0167-7799(97)01076-7

Glossary

C_m ω-x PUFA or m:n(ω-x) PUFA:	Trivial nomenclature: A PUFA containing *m* carbon atoms and *n* double bonds, in which the final double bond is located x carbon atoms (inclusive) from the terminal (ω) carbon atom of the fatty-acid chain.
$m{:}n\Delta^{aZ,bZ,\ldots dZ}$:	Systematic nomenclature: A PUFA containing *m* carbon atoms and *n* double bonds. The superscripts a*Z*, b*Z*, etc. indicate the positions of the double bonds (carbon atoms numbered from the carboxyl end), and that they are of *Z* (*cis*) configuration.
***sn*-2-Phospholipid:**	Trivial nomenclature for phospholipids where *sn* indicates the presence of a chiral glycerol skeleton numbered as *sn*-1, *sn*-2, etc, with the phosphate moiety occupying the *sn*-3 position, and with fatty acid substitution at the 2 (*sn*-2) position.
Δ^m Desaturase:	A desaturase enzyme which introduces a double bond at carbon atom *m*.

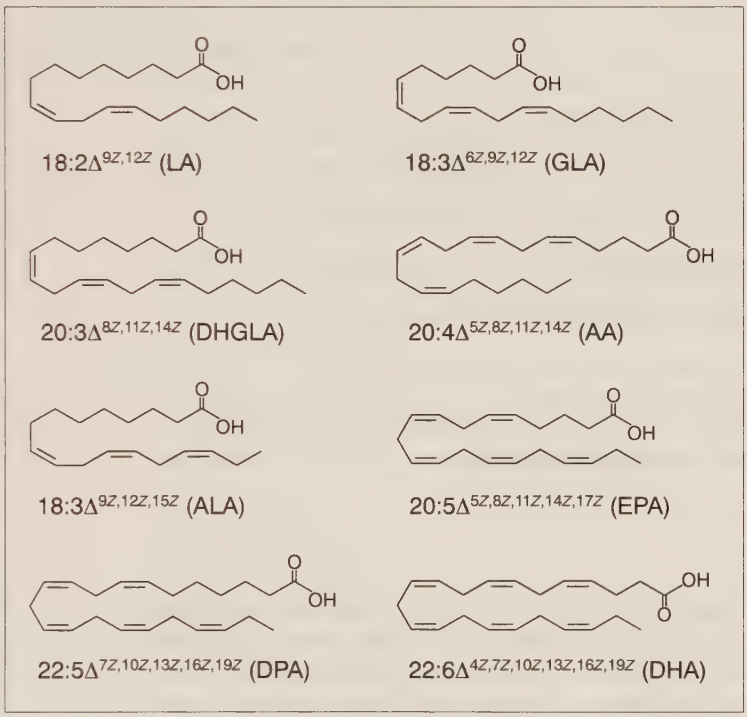

Figure 1
Representative examples of naturally occurring polyunsaturated fatty acids.

microorganisms, especially algae, fungi and bacteria[1,2,7–16]. The acids are typically present in storage oils and membrane lipids, as esters, ethers, glycerides, glycolipids, phospholipids, phosphonolipids, glycosphingolipids, sulpholipids and lipoproteins[1,2,7–16]. PUFAs are biosynthesized via an extension of the saturated-fatty-acid pathway, in which stearate is converted to oleate (18:1Δ^9; see Glossary) and then to linoleate, which is the central precursor for the ω-6 and ω-3 series (Fig. 2)[1,2]. Algae, fungi, bacteria, insects and some other invertebrates possess the array of desaturase and elongase activities required for the *de novo* production of the various PUFAs, and are the primary producers of these compounds in nature[1,2,7–16]. In contrast, higher plants rarely contain PUFAs above C_{18}, owing to a lack of the requisite enzymes, and even GLA and ALA are not very widely encountered. Similarly, higher animals are unable to produce fatty acids over C_{18}, and as they only possess Δ^4, Δ^5, Δ^6 and Δ^9 desaturases (and mammals appear also to lack a Δ^4 desaturase) (see Glossary), they cannot form LA, ALA or longer PUFAs *de novo*. However, they can further elaborate LA (or GLA) and ALA, and so these acids are essential for higher animals, including mammals, which rely ultimately on microbial and plant sources for their provision.

PUFAs are presently obtained commercially from selected seed plants (LA, GLA and ALA), marine fish (AA, EPA and DHA) and certain mammals (AA) (Table 1)[1,2,12,13]. Traditional processing techniques, such as solvent extraction, winterization, urea-adduct formation and distillation, are utilized for refining the oils, although supercritical-fluid fractionation, chromatography and cyclodextrin-complex formation also appear to be useful for the preparation of highly purified acids[1,13,28].

However, with the exception of LA, it has become evident that production from current sources is inadequate for supplying the expanding PUFA market, in terms of both overall output and product quality. These shortcomings arise from several intrinsic drawbacks: (1) present sources are subject to seasonal and climatic variations, and this can result in considerable inconsistencies in oil supply and quality; (2) the lack of sufficient agronomic productivity of the cultivated-plant sources and the limited nature of marine-fish stocks imposes a limit on the extent of exploitation; and (3) the need to refine low-grade oils extensively incurs a large economic cost. The resulting high market price of pure PUFA lipids has handicapped many potential applications. The realization that the PUFA supply will soon be far outstripped by demand, coupled with the inadequate provision of pure polyunsaturated lipids for biomedical usages, has led to the search for alternative bioresources. At present, the genetic modification of oilseed crops and the exploitation of lower plants and animals appear to be the most attractive options.

The engineering of PUFA biosynthesis into oilseeds, with their many agronomic advantages, is one possible route to large-scale production of acids such as GLA, EPA and DHA[17,18,23,35]. Although, ironically, most efforts in this area have focused on suppressing the biosynthesis of polyunsaturates, work is now in progress to introduce and enhance PUFA biosynthetic capacity. The practicality of this has been illustrated with the expression of a cyanobacterial Δ^6 desaturase from a *Synechocystis* sp. in tobacco plants, thereby enabling GLA biosynthesis from LA (Fig. 2)[19]. Together with the availability of algal and higher-plant genes coding for elongases, desaturases and lipidyl

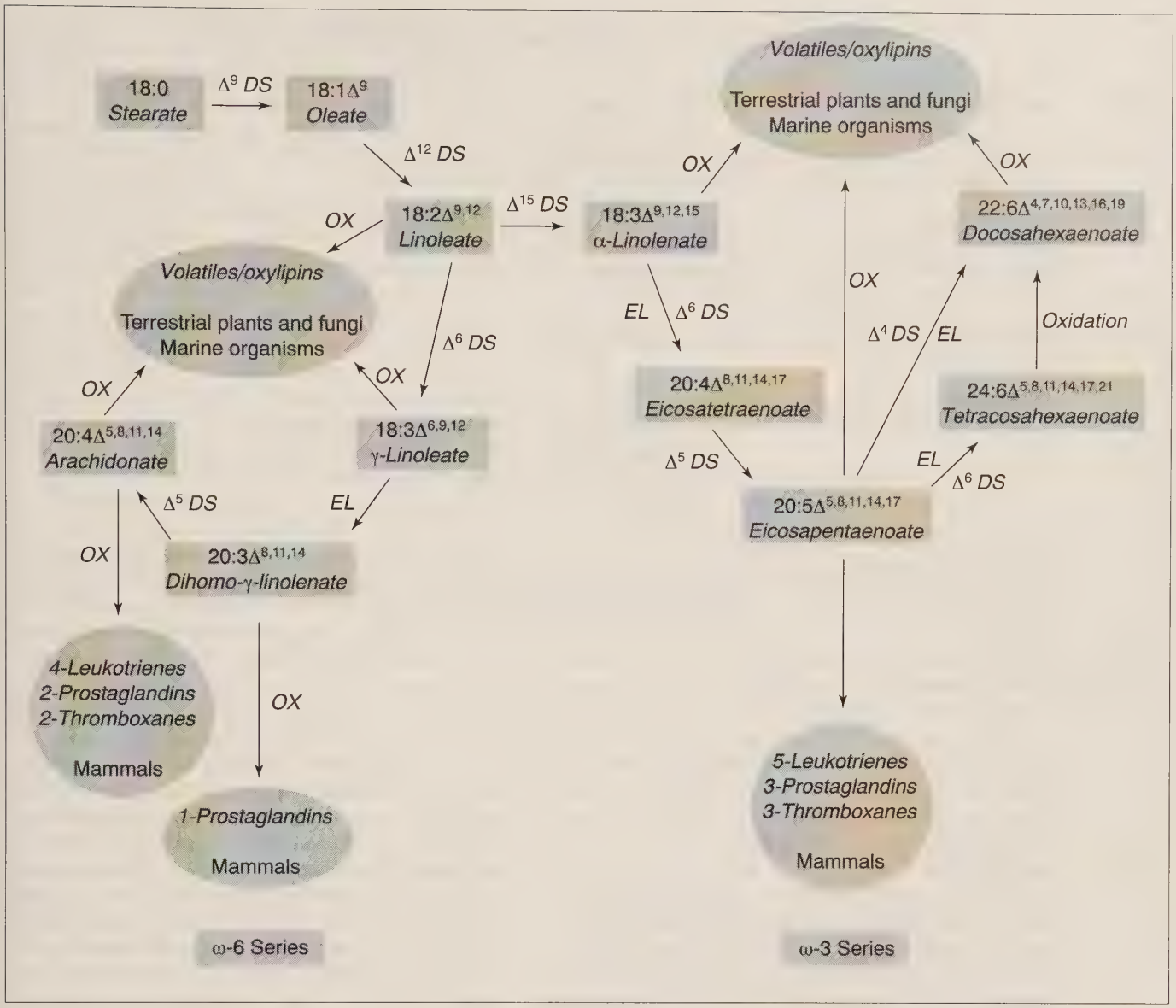

Figure 2

Interconversions of polyunsaturated fatty acids (PUFAs) and their biotransformations into bioactive metabolites in biological systems. Abbreviations: $\Delta^n DS$, *n*-desaturase; *EL*, elongase; *OX*, oxidase enzymes including lipoxygenases, cyclooxygenases, peroxygenases, etc. It should be noted that current evidence suggests that no mammalian Δ^4 desaturase exists and Δ^4 PUFAs such as docosahexaenoic acid (DHA) are thought to arise by the intermediate formation of higher Δ^6 PUFAs followed by chain shortening via peroxisomal β-oxidation.

transferases, this opens up the possibility of producing at least GLA, AA, EPA and DHA in oilseeds, as well as of controlling the type of lipid assembled[17,19]. Some specific benefits can be envisioned for these enhanced crops: (1) a potentially cheap and sustainable supply of PUFAs on a large scale; (2) the PUFA profile could be tailored to meet specific nutritional demands for food oils and feedstock requirements for fine chemicals etc; (3) unusual PUFAs could be produced, as shown by the production of $18{:}4\Delta^{6Z,9Z,12Z,15Z}$ from GLA in tobacco plants containing Δ^6 desaturase activity[19]; and (4) crops expressing polyunsaturates are expected to display enhanced cold resistance, and this could allow for extended cultivation. Programmes have been initiated by Rhône-Poulenc, DuPont, Martek and other agrochemical companies, but this research area is still in its infancy, and the introduction and cultivation of PUFA-producing oilseeds should be viewed as a long-term goal.

The second avenue to surmounting the supply quandry is to harness the unique biosynthetic capacities of lower organisms, which have long been recognized as promising sources, particularly as they can synthesize the entire range of PUFAs and can be grown on an industrial scale[7–16,20–22,24]. For example, terrestrial and marine phycomycete fungi, especially from the *Mucorales*, produce GLA, AA, EPA and DHA[7,8,14–16,20–22,24,26,27,29]. Likewise, uni- and multicellular algae from the *Chlorophyceae* and *Cryptophyceae* are rich in GLA, ALA and EPA, the *Phaeophyceae* and *Rhodophyceae* yield high levels of AA and EPA, and the *Dinophyceae* produce large amounts of EPA and

Table 1. Occurrence of representative ω-3 and ω-6 PUFAs

PUFA	Conventional sources	Alternative sources	Refs
GLA	Plants: *Oenothera, Borago, Ornithogalum* spp. (1.3–7.6%)	Fungi: *Mucor, Mortierella, Aspergillus* spp. (3.6–4.5%) Algae: *Chlorella and Spirulina* spp. (0.6–1.6%)	7–10,14–22
AA	Fish: *Brevoortia, Clupea, Sardina* spp. (0.2–0.6%) Animal tissues (0.1–0.3%)	Fungi: *Pythium, Mortierella* spp. (3.4–6.3%) Algae: *Porphyridium* spp. (1.6–3.6%) Mosses: *Rhytidiadelphus, Brachythecium, Eurhynchium* spp. (0.7–2.4%)	7–10,14–19,21, 23,24
ALA	Plants: *Brassica, Glycine, Juglans, Linum* spp. (4–21%)	Algae: *Chlorella* spp. (0.9–1.7%)	7–10,12–15, 17–19,21
EPA	Fish: *Brevoortia, Engraulis, Sardina, Scomber* spp. (1.7–6.2%)	Fungi: *Mortierella, Pythium* spp. (2.9–6.4%) Algae: *Chlorella, Monodus, Porphyridium, Nannochloropsis, Cryptoleura, Schizymenia, Navicula* spp. (1.8–4.8%) Mosses: *Brachythecium, Eurhynchium, Scleropodium* spp. (0.7–3.1%) Bacteria: *Rhodopseudomonas, Shewanella* spp. (0.6–1.7%)	7–10,13–19,21, 23,25,26
DHA	Fish: *Brevoortia, Engraulis, Sardina, Scomber* spp. (1.6–7.4%)	Fungi: *Thraustochytrium, Entomophthora* spp. (0.9–1.5%) Algae: *Gonyaulax, Gyrodinium, Cryptoconidium* spp. (2.7–4.1%) Bacteria: *Rhodopseudomonas* spp. (0.6–1.1%)	7–10,13–15, 17–19,21,27

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid. The figures in parentheses refer to the overall PUFA content on a fresh-weight basis.

DHA[7,8,14–16,20–22,24,30–33]. Several *Eubacteria*, principally those from marine environments, also synthesize substantial amounts of EPA[7,8,14,15,20–22,24,34].

There are many advantages of such PUFA sources[14–16,20–22,24].

(1) Production can be carried out throughout the year, as there is usually no seasonal or climatic dependence. Bacterial and fungal fermentation technology is also highly developed, and algal and bryophyte cultivation has advanced sufficiently to allow the mass culture of many species.

(2) Environmental and nutritional regimes can be easily controlled, allowing the manipulation of the lipid yield and profile.

(3) Although the lipid contents of these organisms are usually lower than those of conventional sources (2–12% fresh weight rather than 1.6–21%), the oils often contain considerably higher levels of the desired acids (typically 9–80% w/w rather than 7–33% w/w). This simplifies purification, which is a major economic consideration, and can also reduce 'off' flavours and/or odours, which are associated with impurities.

(4) Lower plants and animals are notable for producing PUFA-containing phospholipids, glycolipids and esters, many of which have unique applications in therapeutics.

(5) These organisms are rich in protein, trace elements, vitamins, antioxidants, etc., and can be employed as macro- and micronutrient sources in food formulations.

(6) Genetic transformation protocols are available for bacteria, fungi and algae, thereby enabling the development of overproducing strains and the manipulation of the PUFA profile by pathway engineering.

A number of recent advances have enabled many processes to attain commercial feasibility, notably through the use of overproducing strains, selective inhibition of enzymes, improvements in cultivation regimes and bioreactor engineering. The indications are that a stable economic niche now exists for fungal and algal GLA, AA, EPA and DHA, and that these acids will become available on a multitonne scale in the near future. For example, Rhône-Poulenc, Gist Brocades, Suntory, Martek and Lion have processes in place for synthesizing lipids containing GLA, AA and DHA, using *Mucor* spp., *Mortierella* spp. and *Pythium* spp. Similarly, phototrophic and heterotrophic algal systems employing *Chlorella* spp., *Spirulina* spp. and *Porphyridium* spp. are being developed by Martek, Nisshin Oil Mills, Kelco International, Omega-Tech, Scotia Pharmaceuticals and Héliosynthese for the production of AA, EPA and DHA. Also, companies including Sagami and Nestlé are examining bacteria such as *Rhodopseudomonas* spp. and *Shewanella* spp. and mosses such as *Eurhynchium* spp. and *Rhytidiadelphus* spp. for the production of EPA, AA and DHA. Despite the lack of economic subsidy from government agencies, the necessity of establishing markets and consumer acceptance and the continuing need to improve yields and cultivation techniques, it is clear that unique opportunities exist in the marketplace for 'biotechnological' PUFAs from lower organisms[14–16,20–22,24]. In the short term, these products are expected to target mid- to high-value applications (such as fortified foods, nutritional supplements, health foods and therapeutics) and to provide pure lipids for biomedical research. The majority of lower animals and plants

have yet to be screened for lipid production and future explorations will undoubtedly yield organisms with higher oil contents or that overproduce particular PUFAs, and therefore also expand the available genetic resources for transgenic production.

Biological functions

PUFAs fulfill many structural and functional roles and are unequalled amongst fatty acids in the wide spectrum of biological events in which they participate. First, PUFAs are critical components of cell and organelle membranes (mainly as *sn*-2 phospholipids), as suggested by the close link between organelle evolution in eukaryotes and the emergence of polyunsaturated acids[2,5,35–37]. They are crucial for regulating the architecture, dynamics, phase transitions and permeability of membranes and for the control of membrane-associated processes[2,35–38]. Moreover, they regulate membrane-bound proteins such as ATPase, transport proteins and histocompatibility complexes, and modulate interactions with extracellular components such as fatty-acid-binding proteins[35–38]. In addition, PUFAs also regulate the expression of certain genes, including those coding for fatty-acid synthase, nitric-oxide synthase, sodium-channel proteins and cholesterol-7-α-hydroxylase, and thereby affect processes including fatty-acid biosynthesis, cancer induction and cholesterol regulation[39]. In this way, they have an impact on cellular biochemical activities, transport processes and cell-stimulus responses, are implicated in physiological processes including lipid metabolism and targeting, immune responses and cold adaptation, and are involved in pathological conditions such as carcinogenesis and cardiovascular disease[2,5,35–39].

The second, and more important, function of PUFAs is the provision of a unique class of precursors for conversion into metabolites that regulate critical biological functions (Fig. 2). In plants, they are transformed by a variety of oxidases, cyclases, lyases, reductases and isomerases into oxygenated molecules, ranging from simple aldehydes to oxylipins such as hydroxy- and epoxy-PUFAs, jasmonates and eicosanoids[40,41]. These act as anti-infectives, wound-response mediators, regulatory, hormonal and chemotactic agents, and aroma and flavour compounds[40–43]. In lower animals, such as insects and marine invertebrates, oxylipin and eicosanoid metabolites mediate cellular processes and ecological responses including metamorphosis, reproduction, chemotaxis, water and solute transport, immune function and host–parasite interactions[44,45]. In higher animals, the PUFA–eicosanoid system is crucial to developmental and regulatory physiology, and also features in some disease states[2].

It is from the human biomedical viewpoint that PUFA metabolism and eicosanoid function has its greatest importance. Since the finding that AA is converted to prostaglandins in mammals, much research has been carried out to determine the connection between PUFAs and human physiology. The evidence gathered to date shows that these acids, via the mediation of eicosanoids, are fundamental to the proper maintenance of homeostasis and are linked to serious physiological and pathophysiological syndromes[2–6,46]. The eicosanoid pathway in mammals begins with the phospholipase-mediated release of PUFAs from membrane phospholipids and is followed by cyclooxygenase- and lipoxygenase-mediated reactions that give rise to the two major classes of metabolites, the prostanoids (prostaglandins, thromboxanes and prostacyclins) and the lipoxins and leukotrienes[2,3,52]. The ω-3 acids are converted to the 3-prostanoids and 5-leukotrienes, whereas the ω-6 series produce the very distinct 1- and 2-prostanoids and 4-leukotrienes. As AA is usually the most common PUFA in the body,

Table 2. Physiological effects of ω-3 and ω-6 series eicosanoids

System	ω-6 Eicosanoids	ω-3 Eicosanoids	Biological functions regulated
Cardiovascular	PGI_2, TXA_2	PGI_3, TXA_3	Platelet adhesion and aggregation; fibrinogen; serum lipids; endothelial permeability; vasodilation; vasoconstriction; erythrocyte deformability; blood pressure
Respiratory	PGE_2, $PGF_{2\alpha}$, TXA_2, LTC_4, LTD_4	PGI_3, TXA_3	Bronchodilation; bronchoconstriction; inflammatory responses
Gastrointestinal	PGE_2, PGI_2, $PGF_{2\alpha}$, TXA_2, LTB_4, LTC_4, LTD_4	PGI_3, LTB_5	Gastric secretion; gastric cytoprotection; peristalsis; insulin and pancreatic-amylase
Renal	PGD_2, PGE_2, $PGF_{2\alpha}$, PGH_2, PGI_2, TXA_2, LTB_4	PGE_3, PGI_3, TXA_3, LTB_5	Glomerular permeability and filtration; renal vasodilation; renin secretion; sodium excretion
Immune	5-HPETE, 12-HPETE, 15-HPETE, PGE_2, PGI_2, LTB_4, LTC_4, LTD_4	LTB_5	Vascular permeability; cytokine response; PAF production; leukocyte receptor expression; leukocyte degranulation; macrophage and neutrophil aggregation; chemotaxis; epidermal proliferation

Abbreviations: 5-, 12- and 15-HPETE, 5-, 12- and 15-hydroperoxyeicosatetraenoic acid; LTA_n, LTB_n, LTC_n and LTD_n, leukotriene A_n, B_n, C_n and D_n, respectively; PGE_n and PGF_n, prostaglandin E_n and F_n, respectively; PGI_n, prostacyclin I_n; TXA_n, thromboxane A_n. The numerical subscripts refer to the eicosanoid subclass. Data taken from Refs 3, 6, 46, 52 and 53.

the eicosanoids derived from this acid usually predominate in human tissues. The ω-3 eicosanoids are either weak agonists or, more usually, strong antagonists of the potent ω-6 metabolites, and it is the relative levels and interactions of these compounds that determine homeostatic function[46–52]. Some of the regulatory activities of eicosanoids are indicated in Table 2.

Following the seminal work of Dyerberg and Bang on the low incidence of heart disease amongst Greenland Eskimos[54], it has become clear that eicosanoid dysfunctions may lead to illnesses and disorders including cardiovascular, respiratory, gastrointestinal, renal, dermal and immune diseases, cerebral and ocular underdevelopment, and carcinogenesis[3,46–52]. In many cases, these have been attributed to an imbalanced PUFA intake, often identified as a deficiency in ω-3 acids, resulting in the excessive production of ω-6 eicosanoids and/or the insufficient synthesis of the ω-3 metabolites[3,46–53]. In addition, age, stress and illness can have deleterious effects on ω-3 biosynthesis and may exacerbate nutritional deficiencies. Such disturbances can disrupt normal cellular functions, and the inability to restore homeostasis following cell or tissue injury (both of which can initiate a cascade of ω-6-eicosanoid-mediated physiological effects) may lead to reactions culminating in the diseased state if not sufficiently modulated by the ω-3 PUFA-derived metabolites.

Dietary PUFAs impact on eicosanoid production in a variety of ways[46–53]: (1) competition between the ω-6 and ω-3 PUFAs for and regulation of desaturases and oxygenases; (2) competition between the two PUFA classes for introduction into membrane lipids; and (3) regulation of phospholipases A_2 and C. For example, LA binds more strongly than ALA to Δ^6 desaturase, and the affinity of ω-6 acids for the phospholipid *sn*-2 acylases is similarly greater than that of the ω-3 family. Thus, even where ALA intake is high, the more efficient incorporation and metabolism of LA typically leads to higher levels of AA in tissues. Conversely, EPA and DHA are powerful competitive inhibitors of the lipoxygenases and cyclooxygenases, and this accounts for the potency of fish oils in inhibiting ω-6 eicosanoid production while increasing ω-3 metabolite levels. Thus, the relative and absolute intake of PUFAs may have fundamental consequences for eicosanoid biosynthesis and consequently for cellular and tissue functions.

PUFAs in human nutrition and disease

The origins of present-day eicosanoid-related ailments can be traced to the changing face of human nutrition over the past 10 000 years. Several lines of evidence indicate that the dietary ω-6:ω-3 ratio was between 1:1 and 4:1 at the time of human evolution in paleolithic societies[55–57]. Since then, reductions in ω-3 intakes have elevated this ratio to the stage where it is between 10:1 to 30:1 in most modern societies. This can be attributed to the move away from hunter–gatherer nutrition, resulting in a large rise in ω-6 intake from the increased consumption of plant-seed oils and animal products, and to the development of intensive agriculture, which has reduced the ω-3 levels of farmed foods[55–57]. Thus, normal dietary sources of ALA are certain plant-seed oils (linseed, mustard and rape seed), grains and leafy vegetables, whereas EPA and DHA are obtained almost exclusively from fish, shellfish and marine mammals[3,46]; although GLA is normally only a trace food constituent, its precursor, LA, occurs in large amounts in vegetable oils, and AA is readily acquired from meat products[3,46]. As a result, modern diets are almost invariably biased towards ω-6 PUFAs, with the ω-3 acids often seriously lacking.

As the human diet has developed and diversified, one would expect biochemical adaptations in PUFA and eicosanoid metabolism to have ensured homeostatic function in the face of changes in lipid nutrition. This is true for subsistence communities, which have experienced only small changes in PUFA nutrition, but their industrialized counterparts are quite different[55–57] because of the sharp declines in ω-3 intakes that these have suffered in the past 150 years. Indeed, this feature could well be central to the apparent spread of eicosanoid dysfunctions in modern societies.

The importance of a balanced PUFA intake has been recognized by health organizations throughout the world over the past decade, and there is now some consensus that PUFAs should form at least 3%, and preferably 8–23%, of the total lipid intake, and that the ω-6:ω-3 ratio should ideally lie between 4:1 and 10:1[58,59]. However, it is crucial to note that the exact relations between PUFAs and disease states and the possible benefits of PUFA therapies remain highly contentious issues[3,46,53]. Indeed, although the biological effects of eicosanoids are undisputed, the same cannot be said for their PUFA precursors, and most of the diverse pharmacological effects that have been proposed for PUFAs have yet to be proved. Also, the importance of PUFA intakes as compared with other nutritional factors, genetic determinants and environmental influences, in the initiation and progress of diseases is not clear. It is equally important to appreciate that, although ω-3 PUFAs may be useful for treating selected disorders in certain individuals, research demonstrates that there is no single PUFA that will solve all problems, but that an optimal balance of dietary polyunsaturates exists that reflects a physiological homeostasis determined by genetic and environmental factors[2–6,46–53]. For instance, anticholesterolaemic and anti-inflammatory activities have been observed for EPA, DHA and GLA, despite the fact that these acids give rise to antagonistic eicosanoid metabolites[2–6]. Similarly, some studies have shown improvements in inflammatory conditions upon administering ω-3 PUFAs, whilst others have been inconclusive or even contradictory[53]. Indeed, the 1992 British Nutrition Foundation report concluded that ω-3 acids, especially EPA can inhibit the production of ω-6 eicosanoids and lead to undesirable effects, such as the disruption of platelet function[60].

Thus, PUFA therapies may have no detectable clinical effects or, worse, the excessive intake of ω-3 and/or ω-6 acids may result in dangerous disruptions in eicosanoid homeostasis, and this fact should be borne in mind when considering nutritional and therapeutic issues.

However, broad endorsements and measures can be made in order to correct gross deficiencies in PUFA nutrition in populations. Thus, the health benefits associated with the consumption of marine fish have long been expounded to the public by authorities, especially with regard to cardiovascular disease[58–60]. Health agencies have also recommended that infant formulae are fortified with DHA and AA, to counteract possible deficiencies during development, and companies have responded by introducing prenatal and postnatal products enriched with these acids[58,59]. Additionally, a range of PUFA-fortified foods are now available in Europe, Japan, South-East Asia, Canada and the USA, including bakery goods, butter, margarine, yoghurt, milk, fruit juices, meat products, snacks and ready meals[58].

Although consumer acceptance still remains a major obstacle, nutrition education and food fortification are expected to go some way towards addressing the broader deficiencies in PUFA nutrition[58–60]. However, on a cautionary note, increasing PUFA consumption also potentially carries an elevated risk of exposure to toxic oxidation products, which are implicated in carcinogenesis and thrombotic and inflammatory diseases[60]. In particular, there is concern that current intakes of dietary antioxidants are insufficient to cope with an increased polyunsaturate load, and that serious health hazards could be posed by an elevation of *in vivo* PUFA-peroxide production. Hence, any moves designed to increase dietary polyunsaturates must be accompanied by measures to ensure that antioxidant levels are adequately maintained.

Pharmaceutical and nutraceutical applications

Evidence of the possible medical effects of PUFA deficiencies have, coupled with the growing acceptance of 'pharmafoods' (nutraceuticals) by consumers, brought these compounds to the attention of food and pharmaceutical companies, which have been quick to exploit markets in the biomedical and pharmafood arenas[61]. A variety of speciality PUFA lipids are available for medical uses, ranging from antiaging, antithrombotic, anti-inflammatory, anticholesterolaemic and anticancer drugs to immunostimulant and immunosuppresant therapeutics (Table 3), although their efficacies remain to be proved. Unregulated applications for esters, glycerides and phospholipids, such as nutraceutical additives for foods, nutritional formulae and cosmetics ingredients, have also increased, but the most obvious commercial impact of PUFAs has been in health supplements, with a host of plant- and fish-derived GLA, EPA and DHA products now being available in the marketplace for uncontrolled dietary use[61,74].

This brings us to a point of contention in the PUFA arena, namely whether they should be treated as exceptional nutritional products or as pharmaceuticals[61]. As they occur in foodstuffs, there is a strong case for considering them as functional foods; on the other hand, they give rise to potent bioactives, and, in view of the serious disease states that may result from eicosanoid imbalances, there is justification for regarding them as biomedicals. In the absence of sufficient data on the long-term effects of consuming large amounts of purified polyunsaturates, it is desirable to regulate supplements, and perhaps assign them for controlled medical use only.

Another issue that affects PUFA-fortified foods and supplements is the question of product labelling[75,76]. Current legislation in Europe is lax in this respect, especially with regard to nutraceuticals, and this has

Table 3. Biomedical and nutraceutical applications of commercial polyunsaturated lipids

PUFA product	Application[a]	Ref.
GLA/EPA/ETRA/DHA/DPA oils	Antiaging and anti-inflammatory agents for smokers	62
Ascorbyl esters of GLA/ETRA	Anti-inflammatory, antiatherosclerotic, anticholesterolaemic, thrombolytic and anticancer therapeutics	63
EPA and DHA oils	Anti-inflammatory and immunosuppressant therapeutics	64
EPA/DHA/GLA/AA oils	Therapeutics for drug- or alcohol-induced liver damage	65
GLA/ETA/EPA/DHA oils	Therapeutics for intestinal, liver and biliary conditions	66
GLA/ETRA TAGs	Parenternal and enteral therapeutics and nutritionals	67
ALA/EPA/DHA oils	Therapeutics for diarrhoea	68
EPA/DHA oils	Presurgery immunostimulants	69
EPA/DHA/GLA/AA oils	Parenternal and infant food supplements	70
GLA/ETRA/EPA/DHA TGs	Various therapeutics, nutraceuticals and cosmetics	71
EPA/DHA fats/oils	Thrombolytic and antiatherosclerolitic chocolates	72
EPA, DHA and AA oils	Nutraceutical additives for processed foods	73

[a]Most of the biomedical effects ascribed to the above products have as yet to be fully verified in clinical trials.
Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ETA, eicosatetraenoic acid; ETRA, eicosatrienoic acid; GLA, γ-linolenic acid; TGs, triglycerides. EPA/DHA/GLA etc. indicates that the formulation consists of a combination of two or more of the PUFAs cited. In the other cases the product consists largely of a single PUFA.

Table 4. Polyunsaturated-fatty-acid-derived biotransformation products and their potential applications

Polyunsaturated fatty acids	Product	Applications
AA, DHA, DHGLA, DPA, EPA	Free acids, glycerides, phospholipids, phospholipid conjugates, esters	Food fortification, nutraceuticals, health products, food emulsifiers, nutritional biomedicals, cosmetics additives, drug delivery, therapeutics, etc.
	Aldehydes, ketones, alcohols	Food flavours and aromas
	Leukotrienes, prostaglandins, thromboxanes, prostacyclins, oxylipins	Pharmaceuticals – inflammatory diseases, cancer, immune dysfunctions, aging, gynaecology, obstetrics, etc.
ALA, GLA, LA	Free acids, glycerides, phospholipids, phospholipid conjugates	Health products, drug delivery, therapeutics, etc.
	Aldehydes, alcohols, lactones	Food flavours and aromas, fragrances
	Cyclooxoacids, polyhydroxyacids, epoxyhydroxyacids	Agrochemicals: antifungals, antibacterials, plant-growth regulators, etc.
	Hydroxyacids, ketoacids, epoxyacids	Speciality polymers and resins, synthetic intermediates, etc.

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; DHGLA, dihomo-γ-linolenic acid; DPA, dicosapentaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; LA, linoleic acid.

allowed manufacturers to make various, and often unsubstantiated, health claims for products. Although Europe has yet to examine such issues critically and rule on them, the US Food and Drug Administration recently forced the withdrawal of PUFA supplements that were viewed as making unsubstantiated and misleading assertions. However, it is increasingly accepted that PUFAs should be treated as 'foods for the prevention of disease', a category that will allow some types of health claims. Present indications from the Food and Drug Administration and other regulatory bodies are that sufficient scientific consensus now exists to warrant this classification for products such as infant formulas and fortified foods[58,77]. Although the regulatory pathway for foods seems relatively clear, the situation with supplements is quite another matter and needs to be resolved on both the use and labelling fronts.

Despite our limited understanding of PUFA biochemistry and the many therapeutic, nutritional and regulatory issues that remain to be resolved, the market potential for polyunsaturated lipids in the food, health-supplement, cosmetic and pharmaceutical sectors is clear (Table 4). In addition to such 'first generation' applications, it is predicted that high-value PUFA-derived eicosanoids aimed at treating specific disease conditions will appear in the therapeutics market, once biotechnological approaches are in place for their production.

Conclusion

Research over the past 20 years has highlighted the biological roles of PUFAs as progenitors of a large variety of bioactive metabolites with diverse physiological functions. The realization that many of the chronic illnesses that afflict modern societies may be linked to imbalanced PUFA intakes has focused attention on correcting possible dietary deficiencies and developing PUFA-based therapies – indeed, food fortification has reached the marketplace and a range of PUFA pharmaceuticals are also available that are aimed at treating various illnesses. Also, PUFA-containing health supplements are now widely available, despite there being no control over their use.

This explosion of PUFA applications has created an increasing demand for purified PUFA lipids, and the inadequacy of present plant, mammal and fish sources has led to the search for alternatives, especially algal, fungal and bacterial production systems. Microbial processes are now in place, and it is forecast that they will eventually dominate the supply of high-grade PUFAs to the pharmaceutical and food sectors.

Although we are just beginning to comprehend the biological roles of PUFAs, their impacts on disease and the consequences of dietary modification, it is clear that they are critical nutrients, and a general consensus is emerging that food supplementation can affect the incidence and progression of at least some illnesses. Future research will undoubtedly clarify the exact roles and importance of PUFAs in biomedicine and lead to improved nutritional guidelines and therapies. Advances in their incorporation into foods and pharmaceuticals should provide a spectrum of improved pharmafood and biomedical products.

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Drug delivery to the nervous system

Dusica Maysinger and Anne Morinville

Delivery of drugs to the nervous system remains a challenge despite advances in our understanding of the mechanisms involved in the development of neurodegenerative disorders and the actions of neuroactive agents. Drug accessibility to the central nervous system is limited by the blood–brain barrier; although the peripheral nervous system is more accessible than the central nervous system, problems are still encountered, mainly owing to the poor stability and considerable side effects of many neuroactive compounds when administered systemically. Microencapsulation of neuroactive compounds and living cells producing such substances can overcome some of these shortcomings for delivery to the nervous system.

In the treatment of diseases or conditions that result from the lack of simple hormones or peptides, the administration of these compounds in a controlled fashion could provide therapy. Conditions such as diabetic neuropathy, amyotrophic lateral sclerosis (ALS) and Huntington's disease, and possibly also Alzheimer's and Parkinson's diseases, could benefit from this type of approach. Nevertheless, although some of the candidate compounds for treatment have been identified, an adequate means of long-term delivery to the nervous system is still lacking. Only a limited number of small, stable molecules acting on neurons or non-neuronal tissue are available for long-term treatment that can be administered orally. However, peptide drugs and small peptides are easily degraded by proteolytic enzymes and hence cannot reach their site of action when administered in this way. The strategy for the administration of these readily hydrolysable molecules for the treatment of neurodegenerative conditions of the central and peripheral nervous system (CNS and PNS, respectively) can assume two directions – either the defective or absent gene is replaced, with the expectation that the missing compound will then be produced, or the missing compound is provided directly. This article focuses on the latter approach, which can be achieved by direct delivery of the molecule or through the use of living cells that produce the missing substance.

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Drug-delivery systems

'Microcapsules' contain an agent in their centre surrounded by a polymer or a macromolecular carrier. A drug (or other active agent, or a living cell) is in the core of the microcapsule and the surrounding polymer layer can vary in thickness and permeability, and their diameter usually ranges between 1 and 2000 micrometres[1]. Microcapsules are also known as microspherules, spansules, and coated granules, pellets or seeds[1], and can have several separate cores surrounded by a polymer or a mixture of polymers.

'Microparticles' are matrices containing drugs (or other agents) more or less homogeneously distributed (suspended) throughout the entire matrix with no distinct core or envelope. Their dimensions are in the micrometre to nanometre range. 'Microspheres' (nanospheres) represent examples of microparticles (nanoparticles).

Examples of microparticles and microcapsules and other delivery devices containing neuroactive agents or living cells for administration to the CNS and PNS are provided in Table 1.

Targeted drug delivery and the related problems

Two main categories of targeting exist – passive and active. Passive targeting occurs because of the body's natural responses to foreign material; macrophages from the liver and the spleen play the pivotal role in these actions. Small particles are not as subject to reticuloendothelial-system (RES) uptake as larger ones, and polymeric materials can also influence this process – for example, hydrophilic, neutral surfaces are favoured over more lipophilic larger particles. In

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PII: S0167-7799(97)01095-0

contrast, active targeting may involve the attachment of a receptor device or vector molecule to the drug-delivery system. If the target site is the liver or spleen, the drug-delivery vehicle should be designed to activate the complement system in order to promote RES uptake. If the target is another organ, the vehicle must avoid RES uptake and should include a vector molecule that will direct it to this organ. However, this task remains difficult and only limited success has been achieved so far.

Table 1. Delivery of drugs and cells to the central and peripheral nervous systems

Drug-delivery devices	
Microcapsules	Gel forms
Microsphere-matrix type	Collagen gels
Liposomes	Osmotic mini-pumps
Micelles	Adhesive patches
Conjugates	
Living-cell-delivery devices	
Encapsulated cells	Hollow fibres
Injection of dispersed cells	Collagen gels

Liposomes

Since their discovery by A. D. Bangham in the 1960s[2], liposomes have received much attention as drug carriers. Conventional liposomes resemble plasma membranes, consisting of phospholipid molecules with a polar head with two hydrophobic tails, forming a bilayer[3]. In contrast, micellar subunits possess one such tail and form a single layer[3]. Liposomes are biocompatible, nontoxic and biodegradable[2,3,4], and offer the possibility of carrying hydrophobic, hydrophilic or amphiphilic molecules[3]. Despite these numerous attributes, liposomes have not gained widespread use as drug-delivery systems, due to their instability *in vivo*[2,3,5]. Classical liposome preparations, sterically stabilized liposomes and immunoliposomes are all susceptible to clearance by the immune system[2,3,5].

Most work on liposomal applications has centred on the delivery of anticancer agents and has culminated in the liposomal formulation of doxorubicin[3,5]. Recent advances in liposomal formulations include cationic liposomes used to entrap various polynucleotides[6]. In addition, cationic liposomes have been complexed with DNA; these are synthetically based nonviral carriers of DNA vectors for gene therapy[4], but the *in vivo* lifetime of these promising formulations remains to be determined. At present, the working *in vivo* range of liposomes is restricted to the nanometre scale[2] as long-term circulation depends on size: the larger the liposome, the faster the clearance. Because the action of neuroactive agents (for example, neurotrophic factors) is mostly achieved by their coupling to a receptor on the plasma membrane, causing an intracellular cascade of events, the formulation of these molecules for delivery must allow sustained extracellular release. Sterically stabilized liposomes, which are taken up by endocytosis[5], are therefore unsuitable for this purpose. Finally, owing to the often potent nature of neuroactive agents, sustained drug release, entailing extended *in vivo* stability of the delivery system, remains difficult with liposomes. In conclusion, liposomes are presently unsuitable for the long-term administration of drugs to the CNS and PNS unless adequately stabilized.

Microcapsules and microparticles

A delivery device for long-term treatment of the nervous system needs to possess a lifetime that exceeds that of the entity it is carrying, which may be, for example, a peptide molecule or a living cell. Due to the difficulties in penetrating the blood–brain barrier, research into the long-term administration of drugs to the CNS has classically been directed at permanently installed devices. In order to avoid inconvenient insertions and the possible removal of large implants or permanently installed stainless-steel cannulae, several types of injectable drug formulations, including microcapsules and microparticles, have been applied in neuroscientific research, showing some promise for clinical use. On the other hand, systemic or local drug delivery is needed for administration to the PNS, for example in diabetic neuropathy. Regardless of the ultimate goal, one of the major obstacles remains the choice of construction materials; the biocompatibility and controlled rate of drug release of the material are important variables in the selection process. Table 2 lists some examples of materials used for the encapsulation of neuroactive agents.

Polymers that can be used for the preparation of implants or microspheres may be either biodegradable or nonbiodegradable, with both types having enormous potential for drug delivery in the CNS and for use as vehicles for transplanting viable cells[7,8]. Polysulfones[9], poly(acrylonitrile-co-vinyl chloride)[10], ethylene-vinyl acetate (EVA) and hydroxyethylmethacrylate–methyl-methacrylate copolymer (HEMA–MMA)[7,8] are frequently used nonbiodegradable materials. These water-insoluble systems yield capsules that are mechanically and chemically stable and are convenient for cell implantation[7]. The polymer acts as a semipermeable membrane, allowing the diffusion of the neuroactive agent. However, encapsulation of living cells using these types of materials presents numerous difficulties because of toxic effects of solvents on cells, either during the formation of thermoplastic-based capsules or because of residual solvent.

Biodegradable polymers can be either synthetic or natural and may be degraded *in vivo* both enzymatically and nonenzymatically. Their byproducts should be biocompatible, nontoxic and readily excreted. Only a few immunologically tolerable biodegradable polymers are available. Human serum albumin and bovine serum albumin have been extensively used for targeting anticancer drugs, insulin, and monosialoganglioside

Table 2. Examples of materials used for encapsulation[7,9,10,13]

Name	Source	Biodegradability	Comments
Human serum albumin, bovine serum albumin	Natural	Biodegradable	Nontoxic, immunologically tolerable
Collagen, haemoglobin, gelatine, chitosan, alginate and poly-L-lysine	Natural	Biodegradable	Nontoxic, immunologically tolerable, doubtful purity
Poly(lactide-co-glycolide)	Synthetic	Biodegradable	Versatile, controlled drug release, erodable
Polyhydroxyalkanoate	Natural or synthetic	Biodegradable	Thermoplastic, wide variety of repeating subunits, controlled drug release
Ethyl-vinyl acetate, hydroxyethyl-methacrylate–methyl-methacrylate copolymer	Synthetic	Nonbiodegradable	Thermoplastic
Polysulfones, poly(acrylonitrile-co-vinyl chloride)	Synthetic	Nonbiodegradable	Retrieval after implantation
Fumaric and sebacic acids	Synthetic	Biodegradable	Adhesive molecules, increased *in vivo* lifetime, useful for drug delivery to gastrointestinal tract

(GM1). Other natural biopolymers, such as collagen, haemoglobin, gelatine, chitosan, alginate and poly-L-lysine, have been employed as carriers for parenteral administration[11,12,13], but their cost and lack of purity have hindered their use. Therefore, several types of synthetic biodegradable polymers have recently been developed and used for microencapsulation, because their processing conditions, availability and cost can be controlled more efficiently[1].

Among the biodegradable polyesters, which hold considerable promise for the controlled delivery of proteins, are those based on poly(lactic acid) and poly(glycolic acid). Poly(lactide-co-glycolide) (PLGA), commonly used in sutures, provides great flexibility in terms of composition, thus allowing tailored drug release. The biodegradation and tissue reactions of PLGA have been extensively investigated[14,15]. PLGA is a linear polyester prepared by ring-opening polymerization and subsequently purified to yield a polymeric powder with the chemical structure $[\text{-H2C-CO-O-}]_m$ $[\text{-CH(CH3)-CO-O-}]_n$, and it hydrolyses by an acid- or base-catalysed reaction to form lactic and glycolic acids. The degradation properties of PLGA depend on the molar ratios of the two monomers in the polymer chain, the molecular mass, crystallinity, size and shape of the device, and on the implantation site[16]. For example, the time taken for PLGA copolymer to biodegrade can be varied from about 30 days to almost a year[15,17] by adjusting the lactic acid:glycolic acid ratio.

Polyhydroxyalkanoates (PHA) represent another class of biodegradable materials amenable for microencapsulation and are potentially superior to PLGA. These biodegradable plastics are synthesized by a number of bacterial species[18,19]. The majority of PHAs contain from three to six carbon atoms in the polyester backbone, with numerous variations in the R-pendant chain[18]. Most poly-R-(–)-3-hydroxyalkanoic acids are composed of a three-carbon-atom backbone and up to eleven carbon atoms in the pendant chain[18]. The structure for these poly-R-(–)-3-hydroxyalkanoic acids[18,19] is $[\text{-O-CHR-CH}_2\text{-CO-O-}]_n$ where R represents the pendant chain. This variation in repeating units confers a greater flexibility for tailoring the agent-release properties compared to PLGAs. Because they are part of the natural biosynthesis–biodegradation cycle, PHAs have few or no side effects and, under aerobic conditions, they degrade to water and carbon dioxide[19]. The most widely used of these thermoplastic esters is poly(3-hydroxybutyrate), commonly abbreviated to P(3-HB) or PHB. PHB is often employed in conjunction with hydroxyvalerate to form copolymers such as poly(3-hydroxybutyrate)–(3-hydroxyvalerate), P(3HB–3HV). PHAs have been employed to microencapsulate, among other things, anticancer drugs such as 1-(2 chloroethyl)-3-cyclohexylnitrosourea (CCNU)[20] and 2′,3′-diacyl-5-fluoro-2′-deoxyuridine.

Block-copolymer micelles in drug delivery

Traditionally, micellar drug-delivery systems have been formed from small-molecule surfactants and amphiphilic block-copolymer molecules. The incorporation of the drug into small-molecule surfactant micelles is quite low, representing one of their major limitations as successful delivery systems. In contrast, block-copolymer micelles can incorporate agents to a greater degree. Block-copolymer micelles are formed from individual block-copolymer chains containing a hydrophobic block and a hydrophilic block. A variety of shapes and sizes (between 10 and 100 nm) can be produced using technologies developed by Eisenberg

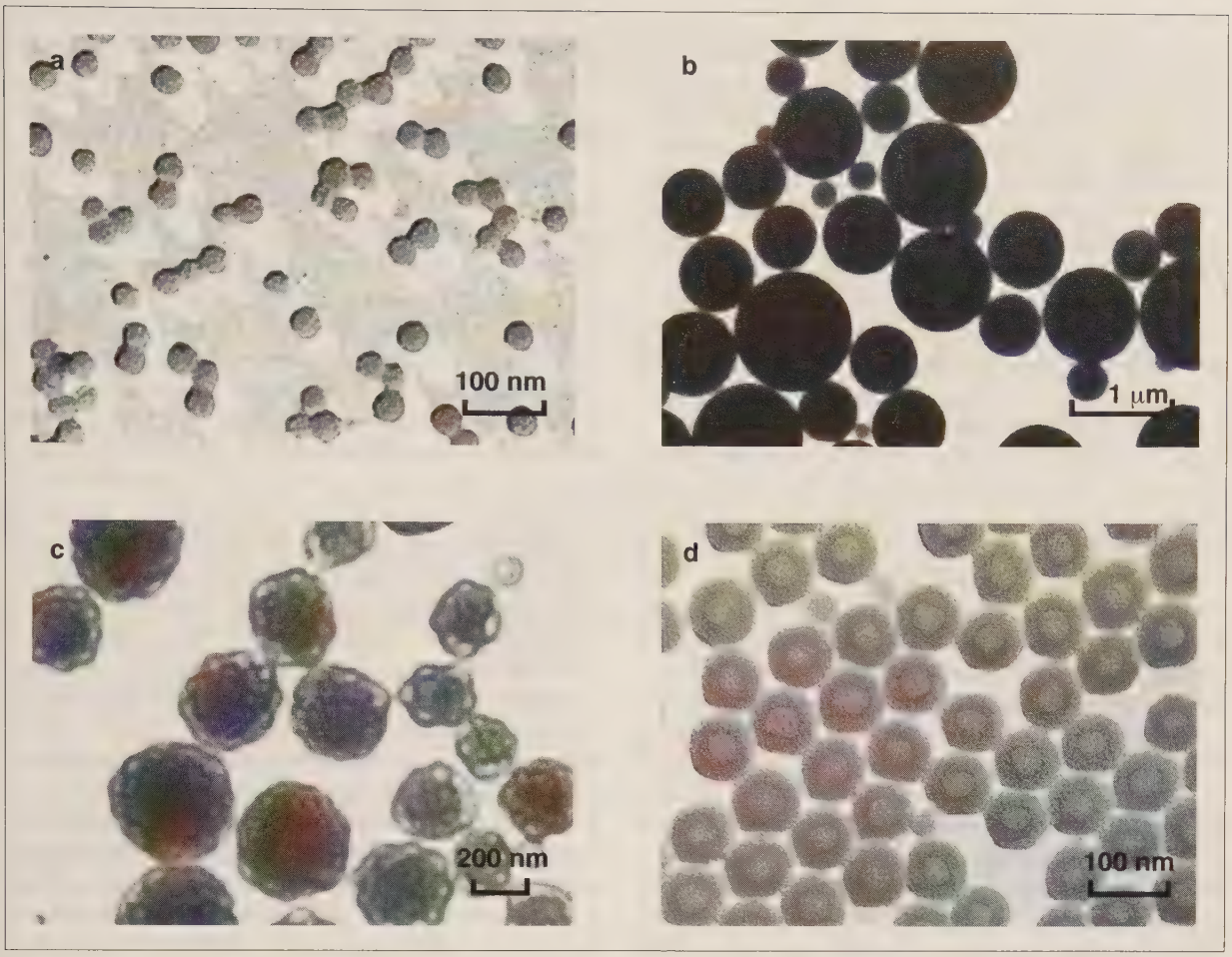

Figure 1

Block-copolymer micelles can be produced in a variety of shapes and sizes as illustrated in panels (**a**) to (**d**). (**a**) Small spherical aggregates [PS(410)-*b*-PAA13 without any additive]. (**b**) Large spheres (compound micelles from the block-copolymer 200-*b*-4). (**c**) Large compound vesicles [aggregates from PS(410)-*b*-PAA(13) with 253 μM HCl]. (**d**) Univesicular aggregates [PS(410)-*b*-PAA(13) with 90 μM $CaCl_2$].

and co-workers[21,22,23] (Fig. 1). These sizes are favourable for avoiding capture by the RES. The amphiphilic character of the block copolymers enables self-assembly of nanometer-scale aggregates with a hydrophobic core and a hydrophilic outer shell in aqueous media. The hydrophobic core accommodates nonpolar drugs and the properties of the hydrophilic shell can be adjusted to both maximize biocompatibility and avoid RES uptake.

Three main groups of block-copolymer delivery systems exist – polymeric drugs, micellar microcontainers and block-ionomer complexes[24]. Polymeric drugs are block-copolymer–drug conjugates in which the drug is covalently bound to the polymer carrier. Common synthetic polymers and copolymers employed as carriers include poly(divinyl-ether-co-maleic anhydride), poly(styrene-co-maleic anhydride) and homopolymers such as poly(L-lysine), poly(ethylene glycol) (PEG) and poly(L-aspartic acid). PEG–polyaspartic-acid block copolymers were used to bind adriamycine, and this system showed several advantages over adriamycine in non-micellar formulation. The major advantages were reduced binding to albumin, greater stability *in vitro*, lower systemic toxicity and apparent higher effectiveness as an anticancer agent. However, the decreased cytotoxicity was caused by the slower release of the covalently bound adriamycine from the polymeric carrier[24].

Recently, the focus has been on micellar systems including noncovalently incorporated drugs, for example micellar microcontainers. These types of micelles are made of diblock and triblock copolymers, and in aqueous solutions they form a water-soluble shell (corona) with a micellar core formed from the hydrophobic loops of polymer chains. The diblock and triblock copolymer micelles are both thermodynamically and kinetically stable in aqueous media and are promising drug-delivery vehicles.

Block-ionomer complexes are formed from amphiphilic block copolymers with a charged hydrophobic core-forming block. The compound to be incorporated into these micelles must have the opposite charge from the core-forming block. When the block copolymer is mixed with a drug, a complex is formed because of electrostatic interactions between the core-forming block and the drug. This kind of micellar block-ionomer complex has recently been shown to be useful for DNA complexing, allowing a novel approach to DNA delivery consisting of the formation of DNA–block-ionomer complexes. Kabanov

et al.[24] demonstrated enhanced stability of 19-mer oligonucleotide incorporated into micelles of poly(ethylene oxide)-*b*-polyspermine in serum. Such types of ionomer-micelle complexes are potentially useful as delivery systems in gene therapy[24]. The therapeutic potential of many drugs, particularly those that are highly liposoluble, could be greatly enhanced by an adequate selection of block-copolymer micelles. The progress in polymer chemistry, in particular block copolymers and rationally designed small peptides that mimic the effects of large neurotrophic proteins, may offer new ways to deliver drugs to the PNS and CNS.

Biologically adhesive molecules

An exciting new range of materials for microspheres are the so-called biologically adhesive molecules. These molecules possess advantages over the other drug-carrier systems, in that they exhibit increased residence time *in vivo* and, hence, the effects of the drug they carry could be sustained for longer intervals. Traditional approaches to the development of biologically adhesive molecules have centred on the use of hydrophilic polymers and hydrogels containing carboxyl groups[25]. Recently, a rapidly degrading hydrophobic polymer consisting of polyanhydride copolymers of fumaric and sebacic acids (FA:SA) exhibited delayed passage through the gastrointestinal tract when compared with other polymers due to an increase in biological adhesion[25]. Poly(FA:SA) 20:80 nanospheres 40–120 nm in size, fabricated using phase-inversion nanoencapsulation, were detected in the cytoplasm of cells and also inside the Golgi apparatus and secretory vesicles[25]. Within the poly(FA:SA) nanospheres, dicumarol, a low molecular weight compound with low solubility and poor bioavailability, displayed increased bioavailability in animals compared with other modes of oral administration[25]. Similarly, orally delivered insulin, encapsulated in a 50:50 blend of poly(fumaric anhydride) and PLGA improved the regulation of glucose in rats in comparison with rats fed an insulin in saline solution or a saline solution alone[25]. These results should, however, be interpreted with caution, as the animals were not diabetic and the usual parenteral administration of insulin was not assessed in comparison with the oral nanosphere formulation. Systemic circulation of these bioadhesive molecules, required for the treatment of many conditions, was not demonstrated. Despite this, these preliminary studies offer exciting possibilities for the oral delivery of a variety of compounds.

Delivery of neuroactive agents

'Neuroactive agents' is a broad term, and includes several categories of peptide, protein and small molecules that act on the nervous system. Neurotrophic agents are substances capable of producing reparation, regeneration or protection of damaged cells in the nervous system. 'Neurotrophic factors' are endogenously produced peptides that participate in the establishment and maintenance of the neural phenotype during embryonic development and adulthood. 'Neurotrophins' comprise a restricted group of neurotrophic factors that are structurally related to nerve growth factor (NGF).

Strategies for delivery

Various approaches have been developed to provide continuous delivery of neuroactive agents, and, although these have overcome some of the problems of delivering these agents, numerous additional problems remain. Even though drug delivery to the PNS appears simpler than delivery to the CNS, problems such as the site of administration, linearity of release, biocompatibility of the polymeric material and loading capacity have still to be solved. Permeabilizers of the blood–brain barrier, osmotic pumps, slow-release polymeric devices and particles, and genetically engineered cells represent some of the tested means of neuroactive-agent delivery. In addition, transfecting neural cells *in vivo* with viral vectors has been extensively investigated and certainly deserves attention (for reviews, see Refs 26–28). Table 3 summarizes some of the advantages and disadvantages of the main categories of nonviral delivery systems. The encapsulation of growth factors and of cells engineered to secrete them is in its infancy; so far, only a limited number have been tested in *in vivo* models (rodents and primates)[9,29–31]. Several recently described examples of neurotrophin administration using microcapsules and microparticles will be summarized here, with an emphasis on those factors claimed to be effective in rescuing or preventing neurodegenerative conditions. Essentially, two main approaches to the delivery of neuroactive agents in encapsulated formulations exist – biodegradable polymeric microparticles and microcapsules, and nonbiodegradable delivery devices such as hollow fibres that accommodate cells synthesizing trophic factors (see Table 1).

Delivery of neuroactive agents using microcapsules and microparticles

Among the most studied neurotrophins are NGF, brain-derived neurotrophic factor (BDNF), neurotrophins 3 and 4 (NT-3, NT-4) and glial-derived neurotrophic factor (GDNF). The physiological roles of these and related neurotrophic factors in nervous-system development and degeneration[8,32,33], the signaling mechanisms related to them[34] and the problems of their delivery to the nervous system[7,8] have been extensively reviewed.

The administration of growth factors such as NGF, insulin-like growth factor (IGF) or human growth hormone (hGH) typically poses many problems, owing to limited oral bioavailability and short *in vivo* lifetimes[35]. A number of human trials involving ciliary neurotrophic factor (CNTF) have yielded disappointing results[36], often because of serious side effects (toxicity)[37]; improvements in drug delivery could lower the therapeutic dose and hence reduce these side-effects[36]. Sustained-release formulations involving microspheres composed of biocompatible and biodegradable polymers could overcome these

Table 3. Properties of common nonviral delivery systems for the peripheral and central nervous systems[8]

Nonviral delivery system	Site of action (central or peripheral nervous system)	Advantages	Disadvantages
Blood–brain barrier permeabilizers	Central	Systemic uptake	High systemic doses, unlocalized brain delivery
Osmotic pumps	Central and peripheral	Cerebrospinal-fluid delivery, long-term delivery	Failure could lead to potentially toxic dumping, refilling of pumps (can lead to infection), parenchymal delivery can cause reaction at delivery site
Microspheres and nanospheres	Central and peripheral	Controlled sustained release, wide choice of materials, potential for implantation of cells, stereotaxic implantation, systemic delivery	Difficult retrieval after implantation, implants containing neuroactive agents need replacement for long-term delivery
Genetically engineered cells	Central and peripheral	Long-term production of neuroactive agent, controlled release	Rejection, therapy with immunosuppressants, low availability of human cells, method of delivery?

shortcomings, and also permit the use of high-toxicity compounds. Recombinant human CNTF (rhCNTF), which has possible applications in the treatment of ALS, was encapsulated in PLGA-and-chitosan microspheres and also in dry alginate with chitosan using the phase-evaporation method[37]. Sustained release was achieved in both cases, with a higher rate of release for the alginate–chitosan microspheres (2–12 days), whereas with PLGA–chitosan microspheres, drug release was observed up to 24 days[37], but this formulation has still to be tested *in vivo*. Colony stimulating factor 1 (CSF-1) has been implicated in the differentiation, proliferation and survival of microglia, but has typically posed delivery problems caused by enzymatic degradation[38]. Recombinant human CSF-1 (rhCSF-1) was encapsulated in PLGA–chitosan microspheres by the solvent-evaporation method[38]. These microspheres potentiated the microglial response to injury; the effect was slightly less when the microspheres were implanted in the peritoneum rather than at the site of lesion[38].

Treatment of diabetic neuropathy could benefit from the encapsulation of neuroactive agents. Reduction of IGF activity has been shown in both human and nonhuman models of diabetes[39]. The expression of IGF-I is controlled by and mimics that of hGH, decreasing after the third decade in nondiabetic individuals[39,40]. Recently, recombinant human growth hormone (rhGH) has been encapsulated in PLGA microspheres using a cryogenic process[35]. In monkeys, injection of these microspheres resulted in an increase of both rhGH and IGF-I for a period of one month[35], even though daily injections of rhGH failed to elicit the same response[35]. Therefore, growth factors within microspheres represent an exciting combination for the potential treatment of neurodegenerative conditions of the PNS.

Living cells for drug delivery

Many neurodegenerative conditions could potentially be treated by the administration of neuroactive agents in a controlled manner through the use of living cells, either wild-type or genetically engineered, that secrete these molecules. However, immortalized cells grafted into the CNS often give rise to tumour formation in the host tissue[7]; the use of primary cells is a possible strategy to overcome this problem. Nevertheless, cell transplantation has typically posed problems due to the low availability of human cells and immunorejection[41], leading to lifelong treatments with immunosuppressants. Immunoisolation offers the advantages of reducing immunorejection and introducing the possibility of xenografts[41]. The immunoisolating device must allow the passage of nutrients and oxygen as well as the active compound of interest, and must be biocompatible with both the host and the transplanted cell. Microencapsulated cells may thus become efficient biological pumps, overcoming some of the difficulties associated with the implantation of dispersed cells or repeated injections of drugs (Table 3). PC12 pheochromocytoma cells and fibroblasts are commonly used in these delivery systems. Encapsulated cells have improved survival and sometimes even produce more neurotrophin than when grown as monolayer cultures[29,30,37]. Anchorage-dependent cells such as fibroblasts need a support to provide long-term survival, and matrigel, collagen and polyelectrolytes (alginate, chitosan) are commonly used inside the polymer capsules.

The use of hollow fibres of nonbiodegradable polymers has the advantage of allowing the device to be retracted when it is not needed or when the cells cease to produce the drug, although surgery would be necessary. In some cases, the administration of neurotrophins or neurotrophin mimetics[42] in biodegradable

microspheres is advantageous, because the polymer will gradually erode, yielding biocompatible degradation products that can easily be eliminated by the body without the need for surgical removal. Technological approaches to and the current status of nonbiodegradable hollow-fibre encapsulation have been extensively discussed in recent reviews[8,43].

Delivery using encapsulated living cells

A variety of devices have been developed for the delivery of living cells, including microcapsules, macrocapsules, hollow fibres, tubular membranes, intravascular devices and flat sandwich pouches[41]. The usual construction materials, such as HEMA–MMA or poly-L-lysine with hardened polyanionic gel, are limited in their utility, because various capsule parameters, such as size, mechanical strength, permeability and wall thickness, are not independent of each other[41]. However, a new method, based on sodium alginate, cellulose sulphate, polymethylene-co-guanidine, $CaCl_2$ and NaCl, has the advantage of allowing independent modifications to the capsule parameters and has recently been employed to encapsulate rat Langerhans islets[41]. This formulation, with living cells transplanted into the peritoneal cavity of mice, produced an effect in both chemically induced diabetic rats and in nonobese-diabetic (NOD) females; these cells functioned for four to six months in the chemically induced diabetic rats and for up to six months in the NOD females[41]. This type of therapy offers the advantage of controlled secretion in response to metabolic demands and signals, thus mimicking the actions of functional cells, without the usual problems associated with transplantation. Nonetheless, the rat islets failed, owing, not to rupture of the capsules or an immune attack[41], but possibly to the low viability of the islet cells themselves, resulting from the insults sustained during their isolation from the pancreas.

No treatment is presently available for Huntington's disease (HD), which is characterized by cognitive and motor deterioration and marked behavioural changes[10]. CNTF has been shown in rodent models of HD to prevent the loss of striatal neurons and to shield against behavioural deficiencies[10]. Baby-hamster-fibroblast cells, genetically engineered to produce human CNTF (hCNTF), were encapsulated in asymmetrical hollow fibres of poly(acrylonitrile-co-vinyl chloride) and implanted intrastriatally into cynomolgus monkeys; deterioration due to HD was imitated using quinolic acid (QA) injection one week after implantation[10]. The animals were sacrificed three weeks after QA injection and the capsules retrieved prior to death. In Nissl-stained sections of the striatum, a significant reduction of lesioned areas in the caudate and putamen was observed in the hCNTF-treated monkeys compared with the controls[10]. Similarly, significant protection of glutamic acid decarboxylase-, choline acetyl transferase- and NADPH-d-positive neurons was also provided by the hCNTF-producing capsules[10]. In addition, atrophy of neurons in layer V of the motor cortex ipsilateral to the lesion was reduced in animals with the hCNTF cells[10]. The retrieved implants exhibited a marked decrease in the rate of secretion of CNTF with respect to the levels measured prior to implantation (12.7 versus 45.1 ng day^{-1} $capsule^{-1}$)[10]. The reason for the decreased production was not determined and the state of the retrieved capsules was not assessed. Death of the encapsulated cells would lead to decreased production; this could be caused by insufficient nutrient availability, an immune response to the cells or a reduction in cell viability due to the encapsulation conditions. Nevertheless, the neuroprotective effect on the cortical neurons innervating the striatum generated by these encapsulated hCNTF-producing cells provides encouraging results for the treatment of HD and other neurodegenerative diseases, with potential applications in Parkinson's and Alzheimer's diseases. However, the feasibility and chronic effects of long-term treatment with these CNTF-producing capsules need to be ascertained.

In addition, CNTF has recently been employed for the treatment of ALS[9]. Baby-hamster-kidney cells, genetically engineered to secrete hCNTF, were encapsulated in hollow-fibre membranes of poly-ethersulfone and implanted into the lumbar intrathecal space of six patients with an early stage of ALS[9]. The production of hCNTF was detected in the cerebrospinal fluid of patients for up to 17 weeks[9], and the devices were removed 13 or 17 weeks following implantation[9]. The major side effects associated with systemic administration of hCNTF were not observed with this form of delivery[9]. However, despite these encouraging results, the disease continued to progress in these patients and long-term studies involving a larger number of patients need to be conducted. Nevertheless, this mode of administration using genetically engineered cells holds promise for the treatment of neurodegenerative conditions. The use of nonbiodegradable hollow fibres, which must be retrieved post-implantation, represents an alternative to microparticles and microcapsules.

Cells from the human embryonic kidney cell line 293, which produces rhCNTF, were encapsulated in biodegradable alginate–chitosan microspheres using an extrusion method without the use of organic solvents[37] and sustained *in vitro* drug delivery was achieved. The use of long-lasting but biodegradable microspheres has the advantage over other invasive devices, such as cannulae and hollow fibres, of not requiring retrieval after implantation[37]. These initial promising results need to be tested *in vivo* to determine the feasibility of these microspheres for the long-term treatment of neurodegenerative conditions.

Recently, LM-10 fibroblast-like cells producing rhCSF-1 have also been encapsulated in alginate–chitosan microspheres[38] and then implanted at the site of a unilateral cerebral cortex ischemic injury or in the peritoneal cavity in mice with no endogenous CSF-1[38]. The microspheres with LM-10 cells potentiated microglial response to the injury, resulting in significant neuron rescue[38]. The survival time of the

LM-10 cells within the alginate–chitosan microspheres can be controlled by varying the physical properties of the chitosan[38]. These results show promise for the administration of cytokines or other neurotrophic factors to the CNS in a sustained-release formulation and for the use of cells producing such factors.

Future prospects

Despite huge advances in the field of encapsulation of proteins and genetically engineered cells, many problems remain to be solved. In the treatment of neurodegenerative conditions, some of the substances that could provide rescue or treatment have been identified, but no suitable means of their delivery is in routine clinical use. Some of these problems could be circumvented by the use of small cyclic peptides, but even these molecules need to be delivered in a suitable manner[42]. In comparison with the more established methods of administering trophic factors, such as repeated injections or via osmotic minipumps, neurotrophin delivery systems based on genetically engineered cells have several advantages. Firstly, neurotrophins stored in a reservoir may be degraded over an extended period of time, whereas cells can be engineered to produce neuroactive agents continually. Secondly, while only a finite amount of neuroactive agent can be stored and delivered in a reservoir, it is possible to adjust the number of cells per sphere in order to modulate the quantities secreted. Thirdly, most pumps are only functional for up to two weeks and have to be replaced if the treatment is required for longer periods of time; this results in greater subject morbidity by, for example, increasing the risk of infection. However, cells can produce neuroactive agents for a much longer period of time and are replaced less frequently; moreover, recent developments in molecular biology suggest that we are now in a position to improve the control of cell function and survival.

Due to the ability to control the release of agents microencapsulated by judicious choice of the materials of construction, this type of technology has the potential for use in a number of areas that require numerous injections, such as vaccination. Recently, oil-based PLGA microcapsules fabricated by the solvent-evaporation method have been described that possess kinetic properties that allowed the release of tetanus toxoid after three weeks or seven weeks[44]. The injection of a cocktail of these microspheres could potentially result in a pulsed release of the tetanus toxoid, mimicking the action of the repeated injections presently required to produce antibodies. *In vivo* studies have still to be performed to assess whether the pulsed delivery from the microspheres will offer immunoprotection[44]. This type of vaccination has the potential to reduce costs and improve patient compliance. Similar kinds of biodegradable microspheres may prove useful for drug delivery to the nervous system.

Many techniques using microcapsules and microparticles have produced sustained drug delivery *in vitro*, and a number of formulations have been tested *in vivo*. Long-term trials of the microparticles and microcapsules *in vivo* have still to be conducted in many cases in order to determine the feasibility of sustained drug release and possible long-term side-effects. Recent progress in polymer science improving block copolymers and lipophilic, adhesive and bioerodable polymers will enable further development of improved delivery systems useful for numerous medical applications.

Some of the problems associated with tissue-specific and site-specific delivery for long-term treatment of neurological disorders could be overcome by administration of multipotent neural progenitors or stem cells. These cells could distribute a therapeutic gene product in a sustained and direct fashion throughout the nervous system. Moreover, they may replace dysfunctional neurons and glia in both a site-specific and global manner, thus offering alternative strategies for treating neurodegenerative disorders[45,46,47].

Acknowledgments

We wish to thank C. Allen for her contribution to the block-copolymer discussion and L. Zhang and A. Eisenberg for providing illustrations of block-copolymer micelles.

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Emerging tandem-mass-spectrometry techniques for the rapid identification of proteins

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State-of-the-art techniques such as liquid-chromatography–electrospray-ionisation tandem mass spectrometry have, in conjunction with database-searching computer algorithms, revolutionised the analysis of biochemical species from complex biological mixtures. With these techniques, it is now possible to perform high-throughput protein identification at picomolar to subpicomolar levels from protein mixtures. This article provides an overview of the techniques and methodologies available for the structural elucidation and identification of proteins and peptides from complex biological samples.

The past ten years have seen an exponential growth in the field of biological mass spectrometry. The inception of such 'soft' ionisation techniques as fast-atom bombardment[1,2], electrospray[3] and matrix-assisted laser desorption ionisation (MALDI)[4,5] in the mid-1980s made it feasible to ionise and introduce into the gas phase thermally labile compounds such as proteins, peptides, carbohydrates and oligonucleotides. These advances in ionisation methods, coupled with various mass analysers, have generated new, technologically sophisticated, mass spectrometry instrumentation that is currently being used to characterise the primary structures of peptides and proteins involved in complex biological processes. These processes, such as antigen processing and presentation, covalent modifications associated with signal transduction and single point mutations, can now be readily studied using tandem mass spectrometry[7–9]. The worldwide large-scale DNA sequencing efforts that are currently being pursued will further aid in the advancement of mass spectrometry to study the complex biological processes[10]. The complete genomic analyses of organisms such as *Haemophilus influenzae, Saccharomyces cerevisiae* and *Escherichia coli*, and the impending complete sequencing of the human genome, will create rich genome-sequence databases[11] that will enhance our ability to study both physiological and biochemical processes. The primary goal of a biological study is to identify the functional species involved in a process, and involves placing a biological context on genes and gene products. Tandem mass spectrometry is ideally suited to provide a powerful link between genomics

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PII: S0167-7799(97)01110-4

and functional genomics (proteome) and create new strategies for the study of biological processes.

Overview of tandem mass spectrometry and its application to peptide- and protein-sequence analysis

Tandem mass spectrometry (MS/MS) has become a very powerful tool in both the analytical and the biochemical sciences. A tandem mass spectrometer consists of two mass analysers separated by an ion-activation device (Fig. 1a). This experimental setup makes it possible to separate an ion of interest using the first mass analyser, induce the ion to dissociate by activation and then analyse the dissociation products in the second mass analyser. These dissociation products provide a 'structural fingerprint' for that particular ion and thereby provide detailed structural information (e.g. amino acid sequence) on it.

Recent years have seen an exponential growth in the application of tandem mass spectrometry for the sequence analysis of peptides and proteins[12–22]. The sequencing of peptides and proteins by various ion-activation techniques has been demonstrated, including low- and high-energy collision-induced dissociation (CID)[12–21], surface-induced dissociation (SID)[22,23] and photoinduced dissociation (PID)[24]. Currently, the most commonly used ion-activation technique for the dissociation of peptide ions is low-energy CID (which involves the collision of peptide ions at low speeds with an inert gas such as argon)[12]. The fragment ions produced upon peptide-ion activation are then analysed by a second mass analyser. These tandem mass spectrometry experiments can also be performed using a single mass analyser, such as a quadrupole ion trap or a Fourier-transform ion-cyclotron-resonance mass spectrometer by separating the mass-analysis and/or ion-isolation events in time[25,26]. Under these low-energy gas-phase collision conditions, peptide ions mostly fragment at the amide (peptide) bonds along the backbone, generating a ladder of sequence ions. If the charge is retained on the N-terminal portion of the fragment ion after cleavage of the amide bond, then b-type ions are formed, and if it is retained on the C-terminal portion, then y-type ions are formed (Fig. 1b)[12]. A complete series of either one or both ion types allows the amino acid sequence to be determined by subtracting the masses of adjacent sequence ions[12,15,23,28–30]. Mechanistic studies have shown that the position of basic amino acid residues such as arginine and lysine on the peptide backbone dictates which type of sequence ion will form; if arginine or lysine is present at the N-terminus of the protein, then the tandem mass spectrum is dominated by b-type ions, and if they are at the C-terminus, then the spectrum is dominated by y-type ions. However, if these basic amino acid residues are somewhere in the middle of the peptide backbone, the complexity of the spectrum is increased. In general, the predictability of peptide-fragmentation patterns in a tandem mass spectrometer under both low- and high-energy collision conditions has led to the development of sequencing methods using these data[12–17].

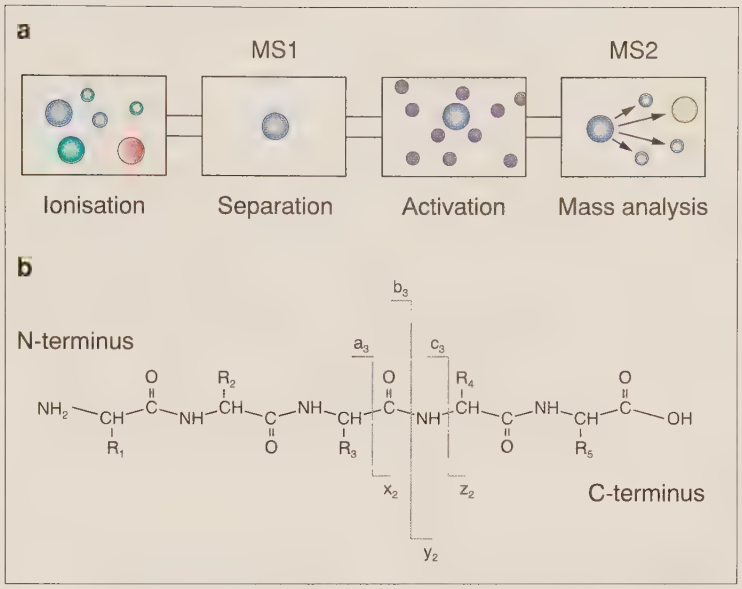

Figure 1

(**a**) Schematic diagram of a typical tandem-mass-spectrometry setup. The two mass analysers, MS1 and MS2, are separated by an ion-activation region, in which the ions of interest (isolated by MS1) are dissociated for analysis in MS2. (**b**) Peptide backbone depicting the typical backbone-cleavage ions. Low-energy ion activation, which is the most widely used ion-activation method, yields mainly 'b_n', 'y_n' and 'a_n' type ions. Ion types 'c_n', 'x_n' and 'z_n' are rarely observed under low-energy collision conditions. The subscript numbers associated with these fragment-ion types indicate the position of the backbone-cleavage site from the N- or C-terminus, depending on the ion type being considered. This nomenclature is a modified form[13] of the Roepstroff and Fohlman nomenclature[17].

Electrospray ionisation: basics and recent developments

The introduction of electrospray ionisation in the late 1980s by Fenn and co-workers created a powerful new method for the ionisation and introduction of biological molecules into gas phase[31], and several reviews on this technique and its uses in peptide and protein analysis have appeared since its inception[32–37]. Electrospray ionisation is a process in which a solution containing the analyte is sprayed across a high potential difference. The charged droplets from the spray are desolvated by either countercurrent gas flow (that is, in the opposite direction to the spray) or by passage through a heated capillary; the ions obtained after desolvation of these charged droplets are then analysed in a mass spectrometer. Over the past ten years, the practice of electrospray ionisation has been refined into a versatile ionisation technique for a wide variety of analytes differing in their chemical makeup, size, complexity and biomolecular stability.

Micro- to nanoelectrospray ionisation

The principal challenge in protein biochemistry is the quantity of protein available for study. Manipulating and analysing minute quantities of protein amidst a variety of complex chemical matrices is a daunting challenge that has led to the modification and improvement of conventional electrospray ionisation. Gale *et al.* have reported the development of a low-flow-rate electrospray (microelectrospray) source that

could efficiently spray small volumes of aqueous solutions, thereby avoiding the denaturing of biomolecules caused by the organic solvents required for the effective performance of conventional electrospray[38]. This source could achieve pressure-assisted flow rates as low as 200 nl min^{-1} with etched fused silica capillaries with inner diameters as small as 20 μm. Improvements in the electrospray signal stability were reported, together with a four- to tenfold improvement in sample sensitivity and a two- to fivefold decrease in sample consumption. Another design, by Caprioli and co-workers, achieved similar improvements in sensitivity, signal stability and signal-to-noise ratio[39], and detection limits of 1 femtomole total sample consumed (10^{-15} mole) for small peptides like methionine–enkephalin. At the same time, Wilm and Mann published a detailed theoretical treatise on electrostatic dispersion during electrospray ionisation process[40,41]. They reported that reducing the size of the emission zone at the tip of the Taylor cone would reduce the droplet size to 200 nm or below, which would allow the flow rate to be reduced to 25 nl min^{-1} (an order of magnitude lower than other microelectrospray sources). This source design, commonly known as a nanoelectrospray source, can easily be adapted to many types of mass spectrometers. These source designs can also be beneficial when small quantities of protein and peptide mixtures (typically 1–2 μl of a solution) can be used for extended periods of time (several tens of minutes) to perform a variety of

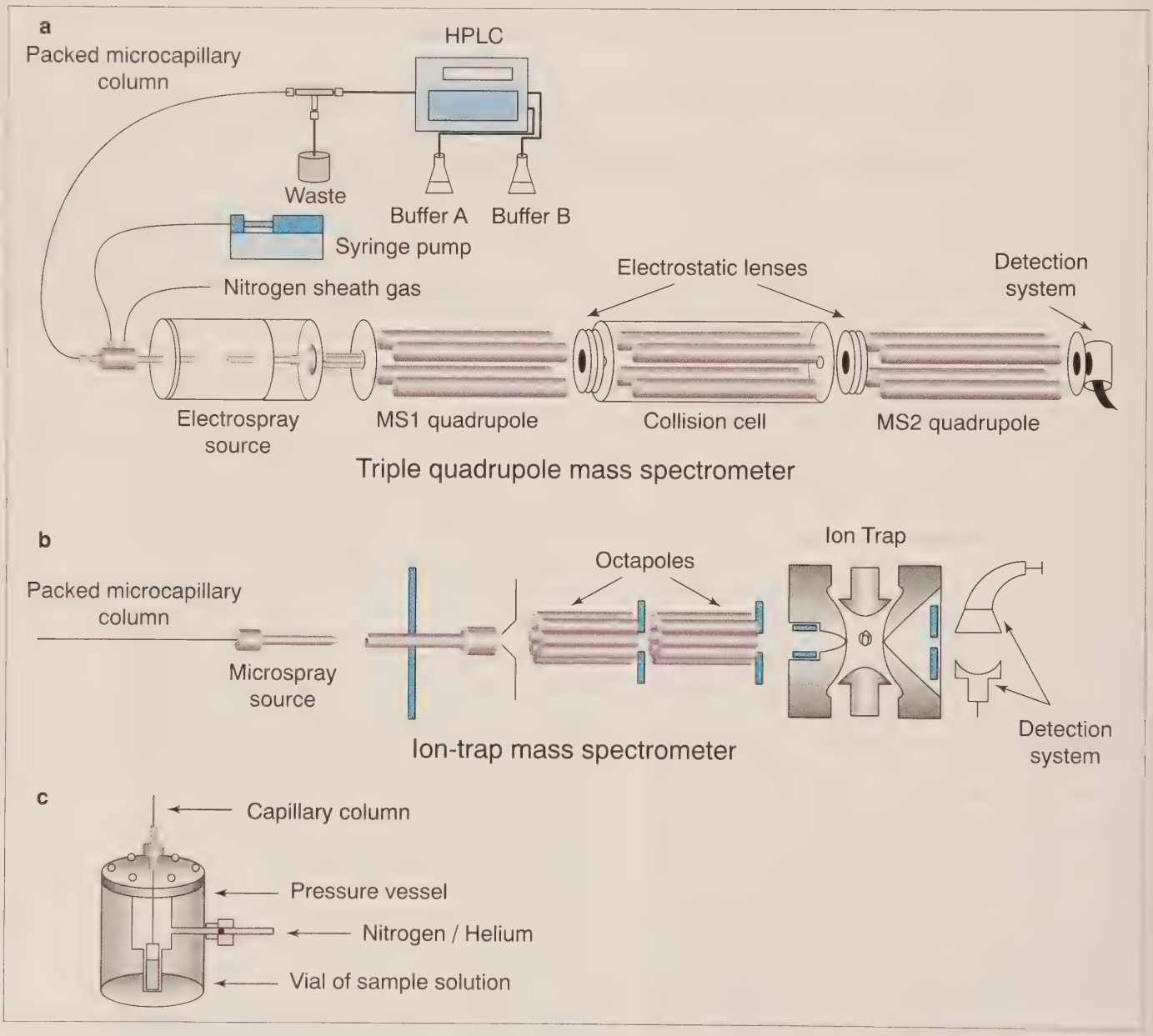

Figure 2

(**a**) Schematic of microcolumn high-performance liquid chromatography (HPLC) with a precolumn microsplitter interfaced to a tandem quadrupole mass spectrometer with a electrospray ionisation source. (**b**) A similar microcolumn-HPLC setup interfaced to an ion-trap mass spectrometer with a microspray ionisation source. (**c**) High-pressure apparatus for efficient sample loading and packing of microcapillary liquid-chromatography columns.

mass-spectrometry experiments. In addition, this extended analysis time helps to achieve an acceptable signal-to-noise ratio by performing extensive signal averaging on peptides present at low-femtomole levels.

Combining liquid chromatography with mass spectrometry using electrospray ionisation

Mass spectrometry is inherently well-suited for mixture analysis, and this suitability can be further augmented by electrospray ionisation, which enables liquid-chromatographic techniques to be combined with tandem mass spectrometers, making it possible to assign structural identity to individual species in a complex mixture by chromatographic separation based on their chemical properties, followed by a separation based on mass:charge (*m*:*z*) ratios and subsequent structural characterisation in the MS/MS mode.

For most studies on peptides and proteins, the low flow rates used by small-bore liquid-chromatography (LC) columns offer the greatest sensitivity for analysis[42]; in order to achieve these low flow rates, microcolumn LC offers a favorable balance between flow rate, performance, reduced sample loss and ease of column construction. Protocols for column construction are described in detail by Shelley *et al.*[43] and Kennedy *et al.*[44] Interfacing the microcolumn LC to electrospray ionisation is straightforward. Figure 2 illustrates a microcolumn-LC setup on a tandem mass spectrometer; standard off-the-shelf high-performance liquid-chromatography (HPLC) pumps can be used, but, in order to achieve low flow rates of 0.5–1.0 μl min^{-1}, a precolumn splitter is used. An elegant HPLC has been built by Davis *et al.* that uses preformed gradients to circumvent the limitations of flow splitting[45]. The system produces reproducible gradients and allows the use of stop-flow techniques to increase the analysis time of a particular chromatographic peak. Furthermore, when working with capillary columns and very small amounts of sample, conventional sample-loading injection loops can lead to sample losses; in order to minimise these losses, samples can be pneumatically injected directly onto the microcapillary column (Fig. 2c).

In order to obtain primary structural information for peptides, it is necessary to acquire tandem-mass-spectral data on the chromatographic peaks that are eluted from the microcolumn LC onto the tandem mass spectrometer. Two approaches are generally employed to acquire tandem-mass-spectral data for peptides. The first approach requires premeasurement of *m*:*z* values for all the components of the complex mixture, followed by a separate analysis of the same mixture to obtain tandem mass spectra. The shortcomings of this approach include high sample consumption and a requirement for considerable manual intervention, leading to limitations on tandem-mass-spectral data acquisition. The second approach involves automated data acquisition by computer control of the instrument, a process that allows both MS and MS/MS data to be acquired in a single analysis. Some tandem mass spectrometers can be operated through instrument control languages that allow precise and rapid control of all instrument parameters[46–48]. This approach improves the efficiency, reliability and accuracy of data acquisition, which is essential for rapid, high-throughput analysis of complex mixtures of peptides and proteins. A typical computer algorithm for the automated acquisition of tandem mass spectra would involve the following steps:

(1) scan a mass analyser to record *m*:*z* values of ions entering a mass analyser;

(2) search this mass spectrum for an *m*:*z* value based on certain preset guidelines;

(3) select the searched *m*:*z* value and subject it to MS/MS conditions that are calculated from the *m*:*z* value of the selected precursor ion;

(4) acquire tandem mass spectra;

(5) reset the instrument back to step 1.

A tandem mass spectrum will be obtained every time the guidelines in step 2 are fulfilled. A computer algorithm based on these steps, when used with a tandem quadrupole mass spectrometer, can theoretically generate a tandem mass spectrum every five seconds, and so a single 60 min microcolumn-LC run with automated MS/MS can yield as many as 400 tandem mass spectra from a complex mixture of peptides.

This abundance and complexity of the tandem mass spectra of peptides make data analysis and structural characterisation of peptides by conventional means (i.e. manual structural elucidation and sequencing) almost impossible. In order to circumvent this problem, we have developed a fully automated computer algorithm to search protein and nucleotide databases using a powerful correlation-analysis method to compare sequences from the database to tandem mass spectra[49]. Automated microcolumn-LC–MS/MS followed by computer-assisted data analysis provides a unique tool to perform rapid, high-throughput analysis for accomplishing protein identification and sequence analysis from complex mixtures[50].

Data analysis using computerised search algorithms

There are a number of computer algorithms available to perform peptide-mass mapping by searching protein databases[51]. Most of these computerised search algorithms are based on using single-stage mass-spectral data. The *m*:*z* values of peptides (which in turn yield the M_R of the peptides) analysed from a proteolytic protein digest are used to search protein databases in order to obtain a list of peptides with a matching M_R and their respective protein sources. The potential shortcomings of this approach include difficulties in obtaining enough peptides for unambiguous identification, due to inefficient proteolysis or small quantities of sample, and unexpected *m*:*z* shifts resulting from protein modifications such as phosphorylation and glycosylation. However, there are some elegant scoring algorithms, such as MOWSE, that perform peptide-mass mapping with considerable degree of accuracy[52]. Recent advances in the mass accuracy of TOF (time-of-flight) mass spectrometers have also contributed to improvements in database searching[53].

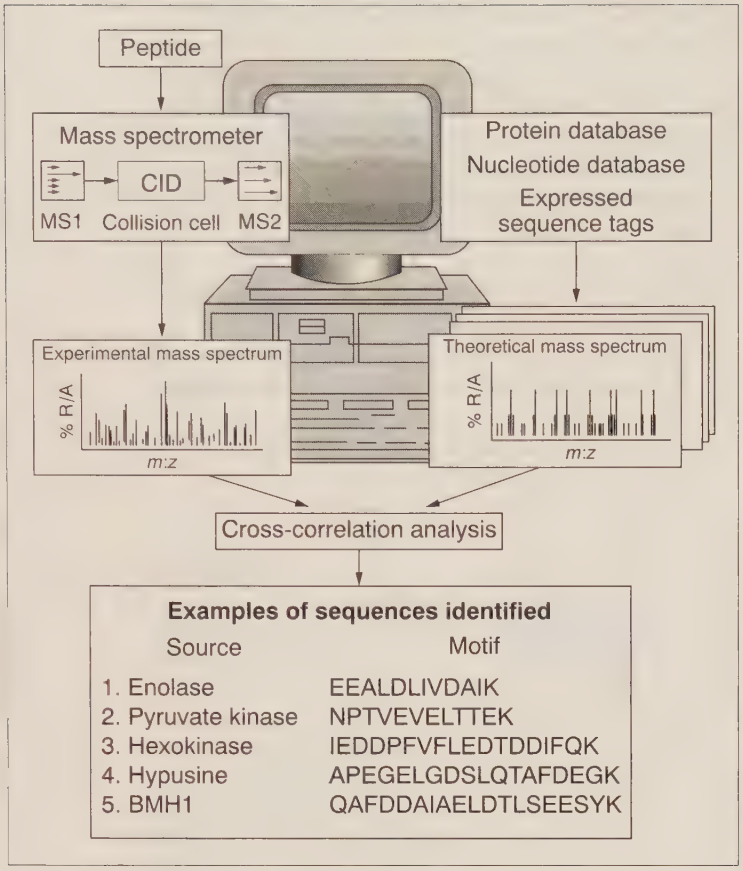

Figure 3

A depiction of the database search algorithm SEQUEST. The experimental tandem mass spectrum is compared with predicted theoretical tandem mass spectra reconstructed from sequences retrieved from protein or nucleotide databases. These sequences are scored against the experimental mass spectrum to obtain preliminary scores. The top 500 theoretical mass spectra from the preliminary scoring are then subjected to cross-correlation analysis; the peptide with highest score is deemed to be the sequence for the experimental spectrum.

Computerised search algorithms for tandem-mass-spectral data analysis

Mass-mapping algorithms are dependent on a set of *m*:*z* values produced by protein proteolysis, and so, for a single peptide or a small set of peptides derived from proteolytic digestion, mass mapping can be ineffective. The use of tandem mass spectra for database searching is ideal for protein identification with a limited set of peptides or to identify a peptide's amino acid sequence. In 1994, our laboratory reported the development of database-searching algorithms using tandem-mass-spectrometry data[49]. Later the same year, Mann and Wilm reported an algorithm for searching databases based on partial sequence information that can be obtained from the tandem mass spectrum of a peptide[54]. The manual interpretation of the tandem mass spectrum produces a short sequence string between three and six amino acids in length, called a 'sequence tag'. This short string sequence, together with the peptide molecular weight, product-ion *m*:*z* values and protease cleavage specificity, are used by the program to generate a small number of potentially matching amino acid sequences. If the search does not identify a sequence, additional searches can be performed by changing the search parameters to exclude 'zones' such as the N-terminal or C-terminal region, signified by the fragment *m*:*z* values. When a fragment ion is removed from consideration, the specificity is decreased and matches to many sequences can occur; this situation requires that all sequence matches be manually evaluated. The use of these sequence tags for database searching has been demonstrated with MS/MS data of intact proteins[55] and also with post-source decay (PSD) MALDI MS/MS data of peptides, but with limited success[56]. Other searching methods using tandem mass spectral data were later introduced that are based on using the *m*:*z* value of the precursor ion, two or more fragment ions and the proteolytic cleavage specificity (http://chait-sgi.rockefeller.edu/ or http://rafael.ucsf.edu/mstag.html); however, unlike Mann's sequence-tag approach, these programs do not require prior manual interpretation of the MS/MS data to identify amino acid sequence.

The second approach developed by our laboratory uses the 'uninterpreted' tandem-mass-spectral data to search protein and nucleotide sequence databases to obtain accurate sequence information about the peptide and/or protein being investigated. This first published report of a computer algorithm for using tandem mass spectra to search sequence databases exploited the predictability of peptide fragmentation in tandem mass spectrometry and is called SEQUEST[49]. The underlying principle of SEQUEST is cross-correlation analysis between the experimental tandem mass spectrum and theoretically generated tandem mass spectra from peptide sequences with a similar molecular weight to the experimental precursor ion (Fig. 3). The peptide sequence with the highest score is deemed to be the one that produced the experimental tandem mass spectrum.

Amino acid sequences in a protein database are scanned to find linear combinations of amino acids that are within a certain mass tolerance of the peptide mass. This mass tolerance can be varied, depending on the requirements of the user, from 0.5 to 200 Da; larger mass-tolerance values lead to slower searches without affecting the accuracy of the search, as two scoring methods are used to evaluate the degree of fit between the peptide sequence and the tandem mass spectrum. The algorithm also has the ability to perform a search based only on the molecular weight of the peptide, or it can incorporate the cleavage specificity for a variety of different proteases that could be used to generate the peptide. Several chemical modifications, including phosphorylation, acetylation and nonstandard amino acid residues such as hydroxyproline and hydroxylysine, can also be considered and incorporated into the search[52]. Nucleotide databases can be searched by translating the nucleotide sequence 'on the fly' (i.e. during the search) to protein sequences in all six reading frames[57].

When an amino acid sequence is within the set mass tolerance, the first (or preliminary) scoring is performed. This considers the predicted fragment ions and matches them to the ions in the experimental tandem mass spectrum. The type of fragment ion

generated is dependent on the type of the tandem mass spectrometer and ion-activation method used, and this program can be tailored to take these differences into account when performing the search. A preliminary score is calculated based on the number of matched fragment ions, the size of the continuous set of sequence ions, the types of immonium ions present in the spectrum and the total number of predicted sequence ions[49]. The correct amino acid sequence is frequently identified purely on the basis of the preliminary score; however, a powerful closeness-of-fit method is used to confirm the highest-scoring amino acid sequence and increase the sensitivity of the search for poor quality MS/MS data. This scoring approach compares the experimental tandem mass spectrum with theoretical tandem mass spectra generated (for the top 500 sequences based on the preliminary score) by calculating predicted sequence ions and then assigning a relative abundance to each ion type; a cross-correlation function is then used to compare the reconstructed spectrum with the experimental spectrum (Fig. 3), and the normalised cross-correlation value determines the 'correct' answer. This two-step scoring method allows full automation of the search process, because no information needs to be derived manually from the tandem mass spectrum. Therefore, in conjunction with microcolumn-LC–MS/MS, SEQUEST provides a powerful approach for performing rapid, high-throughput analysis and protein identification from complex biological mixtures.

Gel-based analysis of proteins by tandem mass spectrometry

1-D and/or 2-D gel electrophoresis are the most popular methods for protein separation and purification, and automated Edman degradation the method of choice for sequencing proteins obtained from gels; the sensitivity of mass-spectrometry techniques has, however, surpassed that of the conventional sequencing techniques. In the past three years, several groups have demonstrated the effectiveness of gel-based protein separation coupled to MALDI–TOF mass spectrometry for database identification of gel-separated proteins with a high level of accuracy and confidence[58–61]. In some examples, the MALDI–TOF mass-spectrometry technique was supplemented by amino acid analysis or PSD of peptide ions to achieve more accurate protein identification. This was mostly due to incomplete digestion of the protein, errors associated with mass assignments due to post-translational modifications and complications caused by cation and matrix-adduct peaks in addition to the protonated tryptic fragments of the protein.

An alternate approach uses the high specificity of tandem mass spectra to identify gel-separated proteins. The general approach is to use MS/MS in conjunction with micro- or nanoelectrospray, microcolumn-LC–electrospray, or microcapillary-electrophoresis–electrospray-ionisation techniques. In 1996, Mann and co-workers reported the characterisation of proteins excised from Coomassie- (50–100 ng protein) and silver- (5 ng protein) -stained polyacrylamide gels by using nanoelectrospray-MS/MS in conjunction with the 'PeptideSearch' program, which uses the sequence-tag approach to database searching[62,63]. They identified and structurally characterised, at the 400 ng level, a 45 kD protein that inhibits the proliferation of capillary endothelial cells *in vitro* and thus may have potential antiangiogenic effects on solid tumors[62]. Furthermore, the utility of this technique as an adjunct to peptide mass mapping has also been demonstrated by identifying various *S. cerevisiae* proteins separated by 1-D and 2-D gels[63]. The sequence-tag approach, combined with MS/MS, has been also used to identify a protein in a pathway associated with apoptosis[64]. Figeys *et al.* have also successfully identified yeast proteins from silver-stained 2-D gels using an integrated solid-phase microextraction and capillary-zone electrophoresis peptide-separation device that was coupled to a tandem mass spectrometer through a microelectrospray-ionisation source, in conjunction with SEQUEST[65]. They have also been able to achieve detection limits as low as 600 attomoles (600×10^{-18} moles) for a tryptic digest of bovine serum albumin using this device[65].

The automated nature of database searching with SEQUEST lends itself to large-scale studies for the rapid identification of proteins separated by 2-D gel electrophoresis. Recently, Link *et al.* performed an analysis of the proteins observed in *H. influenzae* NTCC 8143. From the 303 spots isolated from a 2-D gel, they were able to identify 263 unique proteins[66]. Using an autosampler coupled to a microcolumn-LC–MS/MS in conjunction with SEQUEST [searching tandem mass spectra of peptides from tryptic

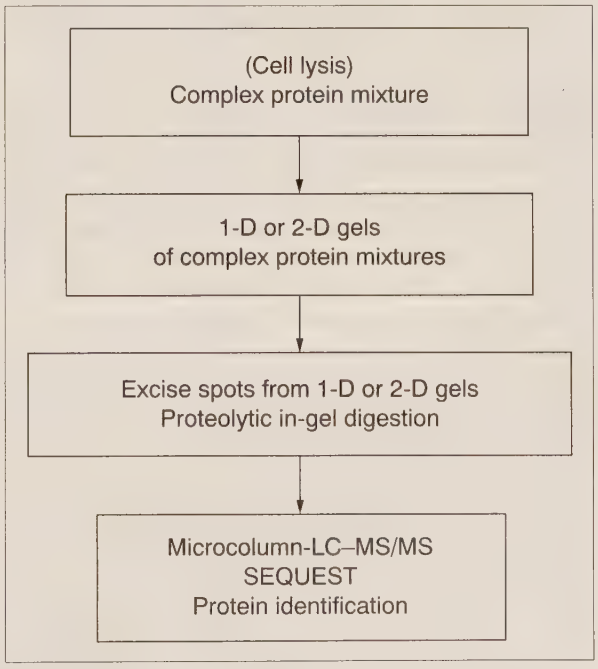

Figure 4
A flow chart to illustrate the steps involved in protein identification from 1-D or 2-D gels by tandem mass spectrometry. Details of the in-gel digestion procedure are elaborated in Refs 41 and 42.

digests against translated genomic sequences (Figs. 3 and 4)] they were able to identify proteins at a rate of 22 per day. This analysis also revealed that about 9% of the sequenced spots were a mixture of two or more proteins, indicating that some proteins will comigrate even during a 2-D electrophoretic separation. These studies also help address fundamental biochemical questions, such as the identities of the most abundant proteins, as well as assessing the similarity between experimental protein sequences and those predicted by gene sequences, correcting several frame-shift errors in the nucleotide sequence and verifying the existence of 25 hypothetical proteins. Interestingly, a protein identified in this *H. influenzae* strain had been deleted from the RD strain that was used to generate the genomic sequence.

Shotgun identification of peptides and proteins from complex mixtures

Identification of proteins from a heterogeneous complex biological mixture without any prior separation, such as 1-D or 2-D gel-based separation, would allow a rapid analysis of multiprotein complexes and protein–protein interactions, as well as a survey of proteins and protein complexes localised to different subcellular locations, with minimal sample manipulation. We are actively pursuing this approach rapidly to identify and thereby identify peptides using fully automated microcolumn-LC–MS/MS and SEQUEST database searching. In this approach, protein mixtures are proteolytically digested without separation and then directly analysed by microcolumn-LC–MS/MS. The techniques and procedures used to achieve direct rapid identification of proteins from heterogeneous protein mixtures have recently been described[50]. This approach is described as 'shotgun identification' because of the obvious similarity to the highly successful approach currently employed in genome sequencing. This type of analysis affords good reproducibility, dynamic range and sensitivity, and has been demonstrated using a test protein mixture with widely varying concentrations and molecular weights. Using the direct microcolumn-LC–MS/MS technique and SEQUEST database searching, we have been able to characterise proteins enriched by immunoaffinity interactions, interactions with glutathione-S-transferase fusion proteins and interactions with macromolecular complexes[50]. Variations of this approach have been successfully applied by McCormack *et al.* to identify antigenic peptides isolated from murine major histocompatability (MHC) class II molecules[47,67] and by Link *et al.* to survey the identities of *E. coli* periplasmic proteins[68]. We are currently using it to pursue the large-scale identification of antigenic peptides isolated from murine MHC-class-II molecule IA^b. MHC-class-II molecules were isolated by immunoaffinity purification and the bound peptides eluted and identified using microcolumn-LC–MS/MS. Approximately 150 antigenic peptides, constituting approximately 50–60 endogenous proteins, were identified (Dongré, A. R. *et al.*, unpublished). We are also currently identifying protein fragments present in the endosomal and lysosomal compartments of antigen-presenting cells using this approach, in order to understand the mechanisms of formation of MHC-class-II complexes and their trafficking from the endoplasmic reticulum to the cell surface (Dongré, A. R. *et al.*, unpublished).

Future prospects

The ability to identify proteins rapidly and accurately by using mass spectrometry has been an unexpected benefit of the genome projects. Mass spectrometry, and, in particular, tandem mass spectrometry, will be an important tool for high-throughput functional studies of the gene products. Protein identification using tandem mass spectra has several important advantages. First, an identification is possible on the basis of a single peptide spectrum. Second, each tandem mass spectrum represents an independent piece of information, and so additional spectra that match the same protein add considerable strength to the identification. Third, the ability to identify proteins based on a single tandem mass spectrum allows the identification of proteins present in complex mixtures. Finally, post-translational modifications do not appear to complicate the identification and can be placed within the amino acid sequence at the specific site of modification with the aid of computer programs. In the not-too-distant future, most protein-biochemical studies will benefit from the ability to identify proteins rapidly by using mass spectrometry.

Acknowledgments

The authors would like to acknowledge the financial support provided by grants from National Institutes of Health (R01GM 52095) and National Science Foundation, Science and Technology Center (BIR 9214821). A. R. Dongré is supported by a Howard Hughes Medical Institute Research Associateship. The authors would also like to acknowledge important suggestions made by A. Y. Rudensky.

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Applications of dielectrophoresis in biotechnology

Ronald Pethig and Gerard H. Markx

Recent progress in the development of microelectrode structures has led to new techniques for the dielectrophoretic characterization and sorting of cells, microorganisms and other bioparticles using nonuniform AC electric fields. These methods utilize differences in the dielectric polarizabilities of cells for their effectiveness, and factors controlling such properties include the conductivity and permittivity of membranes and any cell walls, electrical double layers associated with surface charges, cell morphologies, and internal structures. Applications of dielectrophoresis have included the selective spatial manipulation and separation of mixtures of bacteria, viable and unviable cells, cancerous and normal cells, and red and white blood cells.

The term dielectrophoresis (DEP) was first used by Pohl[1,2] to describe the motion of particles caused by dielectric polarization effects in nonuniform electric fields. Unlike electrophoresis, in which motion is determined by the net intrinsic electrical charge carried by a particle, dielectrophoretic motion is determined by the magnitude and polarity of the charges induced in a particle by the applied field. Furthermore, these induced charges impart the properties of an electric dipole to the particle. There is also an important experimental difference, in that electrophoresis involves the application of direct current (DC) or low-frequency electric fields that are usually homogeneous, whereas for dielectrophoresis, alternating current (AC) fields of a wide range of frequencies are used and the fields must be inhomogeneous.

The basis of dielectrophoresis

The basic dielectrophoretic effect is demonstrated in Fig. 1, in which electrodes of spherical geometry are used to generate an inhomogeneous (nonuniform) electric field. Many different electrode geometries have been used in DEP applications, but the spherical type is used here as the model system because the nonuniform field it generates can be described by a simple formula[3,4]. The dipole moment m induced in the particle can be represented by the generation of equal and opposite charges (+q and –q) at the particle boundary. The magnitude of the induced charge q is small, equivalent to around 0.1% of the net surface charge normally carried by cells and microorganisms, and can be generated within about a microsecond[3]. The important fact is that this induced charge is not uniformly distributed over the bioparticle surface but creates a macroscopic dipole.

If the applied field is nonuniform, as in Fig. 1, the local electric field E_l and resulting force ($E_l.\delta q$) on each side of the particle will be different. Thus, depending on the relative polarizability of the particle with respect to the surrounding medium, it will be induced to move either towards the inner electrode and the high-electric-field region (positive DEP) or towards the outer electrode, where the field is weaker (negative DEP).

Following established theory[2–5], the DEP force F_{DEP} acting on a spherical particle of radius r suspended in a fluid of absolute dielectric permittivity ϵ_m is given by Eqn 1:

$$F_{DEP} = 2\pi r^3 \epsilon_m \alpha \nabla E^2 \qquad (1)$$

where α is a parameter defining the effective polarizability of the particle and the factor ∇E^2 is proportional to the gradient and the strength of the applied electric field. The polarizability parameter α varies as a function of the frequency of the applied field and, depending on the dielectric properties of the particle and the surrounding medium, can theoretically have a value between +1.0 and –0.5. The value for α at frequencies below 1 kHz is determined largely by polarizations associated with particle surface charge, while with increasing frequency, first the effective conductivity and then the effective dielectric permittivity become

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PII: S0167-7799(97)01096-2

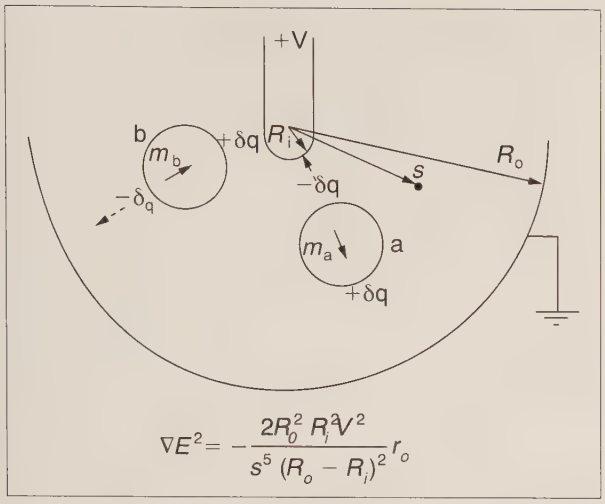

Figure 1

Two particles located in a nonuniform electric field, generated in this example by electrodes of spherical geometry and radii of curvature R_i and R_o. Different dipole moments *m* (depicted as separated microcharges q) are induced in each particle. Particle *a* is more polarizable than the surrounding medium, and is directed by positive dielectrophoresis towards the high field at the inner electrode, whereas particle *b* is weakly polarizable, and moves towards the low-field region at the outer electrode under the influence of negative dielectrophoresis. The force causing this motion is proportional to the factor ∇E^2, described by the equation[3,4], where *s* is the distance of the particle from the inner electrode, *V* is the applied (rms) voltage and r_o is a unit-radius vector.

the dominant contributing factors[3]. A positive value for α leads to an induced dipole moment aligned with the applied field, as shown for particle *a* in Fig. 1, and to a positive DEP force. A negative α results in an induced dipole moment aligned against the field, as for particle *b* in Fig. 1, and produces a negative DEP. The fact that the field appears as ∇E^2 in Eqn 1 also indicates that reversing the polarity of the applied voltage does not reverse the DEP force. AC voltages can therefore be employed and, for a wide range of applied frequencies (typically 500 Hz to 50 MHz), the dielectric properties of the particle, as embodied in the parameter α, can be fully exploited.

Equation 1 represents the conventional dipole approximation of DEP, and is valid providing the field is not too inhomogeneous, as can occur very close to electrode edges. Also, with some electrode geometries, regions of zero electric field are generated, in which the net dipole moment induced must therefore also be zero. In such cases, a general multipolar theory must be used to describe the DEP force accurately[5].

When a particle is suspended in water it will experience either a buoyancy or a gravitational settling force and, if small enough, a sizeable randomizing force caused by Brownian motion. For a particle of radius 1 μm and density 1.05 kg m^{-3} (i.e. typical of a cell or a latex bead) suspended in an aqueous medium at room temperature, the gravitational and Brownian forces each have a value around 2×10^{-15} N. To have an appreciable influence, the DEP force should be at least ten times larger, which, from Eqn 1, requires the factor ∇E^2 to be around 9×10^{12} $V^2 m^{-3}$ for $\alpha = 0.5$. For larger particles, because the DEP and gravitational forces are proportional to the particle volume, this required value for ∇E^2 is independent of the particle radius. However, for submicrometre particles, the randomizing Brownian effect (being proportional to $1/r$) rapidly exceeds the gravitational influence (being proportional to r^3) and becomes the dominant competing force for DEP (Fig.2).

The advantages of using microelectrodes

In the early work that demonstrated the phenomenon of dielectrophoresis and its potential biotechnological applications, the electrodes took the form of metal sheets, wires, rods and pins[2]. In other words, the electrodes were considerably larger than the size of the bioparticles. Following the introduction of microelectrodes to this field of study[6–8], using photolithography and associated semiconductor microfabrication technologies, dielectrophoresis has developed into a technique that can be incorporated into 'biolaboratory-on-a-chip' devices[9].

The advantages to be gained by reducing the scale of the electrode design can be illustrated using the example of the spherical geometry shown in Fig. 1, for the case of a particle located one-tenth of the distance from the inner to the outer electrode. Fig. 3 shows how the values of the electric field and the factor ∇E^2 at this particle location vary as the electrode scale is reduced while maintaining the applied voltage at 1 V rms (root mean square). Reducing the radius of curvature 100-fold produces a 1000-fold increase in ∇E^2. This can be viewed another way, by noting that the factor ∇E^2 has units of $V^2 m^{-3}$. Thus, from straightforward dimensional analysis, an *n*-fold change in electrode scale, for any electrode design, results in an operating voltage change of $n^{3/2}$. For a 100-fold reduction of electrode size, a 1000-fold reduction of operating voltage will therefore produce the same DEP force on a particle in the same relative location.

In addition to the practical advantage of being able to use lower operating voltages for a given desired DEP force, there is also a significant reduction in electrical heating and electrochemical effects. The energy deposition from the field is proportional to σE^2, where σ is the conductivity of the suspending fluid. In the example just given, for a 100-fold reduction of electrode scale, because there is a ten-fold reduction in the applied electric field strength, the electrical heating is reduced 100-fold. Also, as the surface area of the electrodes in contact with the fluid is decreased, surface electrochemical processes are reduced[10]. As the particle radius is reduced below 0.1 μm, values for ∇E^2 greater than 10^{17} $V^2 m^{-3}$ are required for the DEP force to be ten times larger than the Brownian force. Figures 2–4 indicate that this is possible by using microelectrode structures and higher applied voltages, but the corresponding field strengths required will exceed 10^6 V m^{-1}. If the conductivity of the suspending medium is larger than around 0.1 S m^{-1}, such high

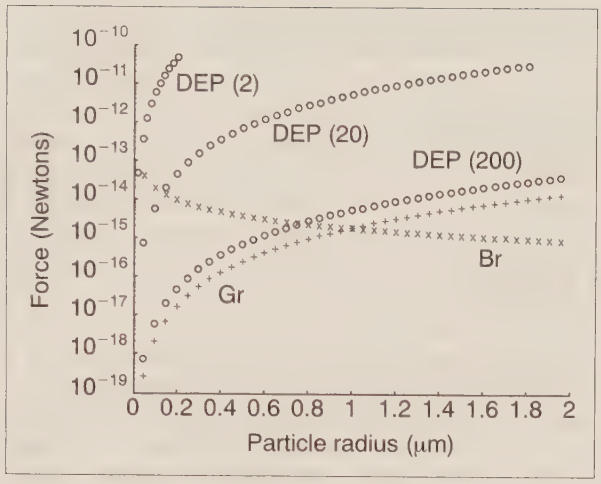

Figure 2

A comparison of the dielectrophoretic (DEP), gravitational (Gr) and Brownian-motion (Br) forces exerted on a particle of density 1.05 kg m^{-3} suspended in water, as a function of its radius. The particle is located one-tenth of the distance from the inner to the outer electrode, with the same spherical geometry ($R_i = R_o/100$ where R_i and R_o are the radii of the inner and outer electrodes) shown in Fig. 1, and DEP forces are shown for R_o of 200, 20 and 2 μm. The applied voltage is 1 V rms.

field strengths can create hydrodynamic effects as a result of localized heating of the fluid. Müller *et al.* have used submicron-scale electrodes to achieve the stable DEP levitation and trapping of particles as small as 14 nm, but thermal gradients and hydrodynamic effects (manifested as fluid streaming) were reported to influence this effect significantly[11]. Finally, microelectrodes used for DEP separation of bioparticles often have a periodically repeating interdigitated geometry. As shown in Fig. 4, the values for ∇E^2 generated by such electrodes decay exponentially with distance from the electrode plane, at a rate inversely proportional to the characteristic electrode periodicity. This information is important for determining the stress exerted on bioparticles or, for example, in the design of electrode chambers that combine dielectrophoresis with field-flow fractionation (FFF)[12].

Dielectric properties of cells

Biological cells exhibit large induced-dipole moments that are highly dependent on the frequency of the applied field[4,13]. This is caused by the fact that cells consist of adjacent structures of materials that have very different electrical properties, leading to large (frequency-dependent) interfacial polarizations at the boundaries between the structures. For example, the cell membrane consists of a lipid bilayer containing many proteins, and is not only very thin but also highly insulating. The inside of a cell is complex and can contain not only membrane-covered particulates, such as mitochondria, vacuoles and a nucleus, but also many dissolved charged molecules. Thus, whilst the conductivity of the cell membrane tends to be around 10^{-7} S m^{-1}, that of the interior can be as high as 1 S m^{-1}, a difference of some 10^7. On death, the cell membrane becomes permeable and its conductivity increases by a factor of about 10^4, with the cell contents freely exchanging material with the external medium. This large change in the dielectric properties on cell death shows up as a large change in the dielectric polarizability. Small changes in membrane properties can also be detected using DEP and used for the selective separation of live or dead cells. Because cells of different species, stages of differentation or physiological state can have widely different morphology, structure or composition, it is not suprising that this also results in differences in their polarizability and hence DEP response.

Typical DEP spectra of different cell types (e.g. plant[14], mammalian[15], bacterial[16], yeast[17]) are shown in

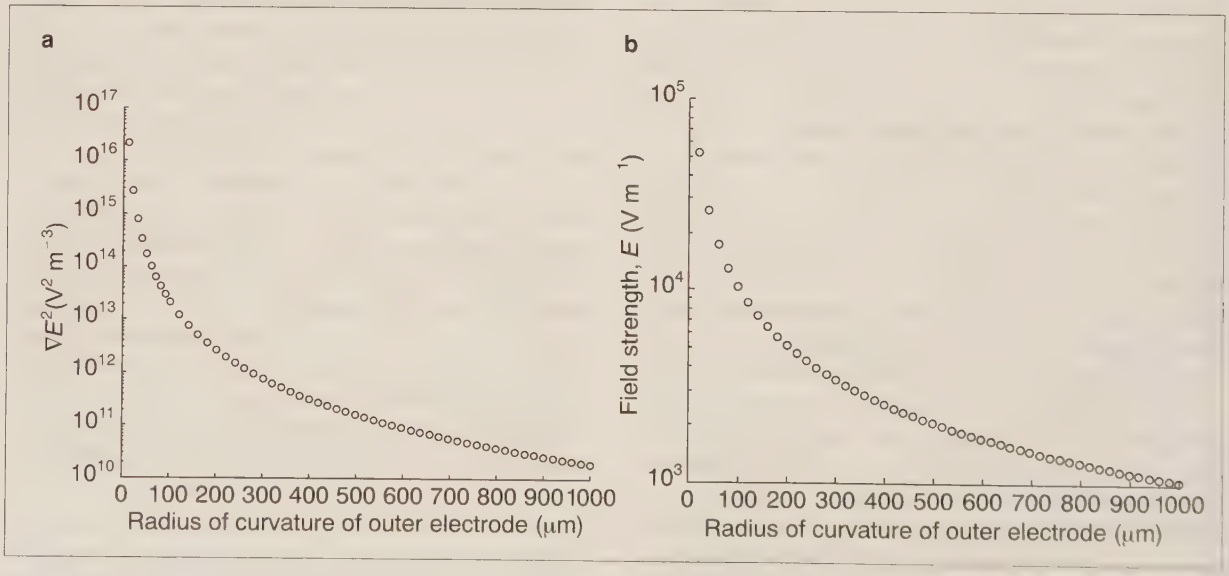

Figure 3

The value of the factor ∇E^2 (**a**) and the field E (**b**) at a particle location one-tenth of the distance from the inner to the outer electrode of the spherical geometry shown in Fig. 1, as a function of the radius of the outer electrode, with a fixed applied voltage of 1 V rms.

Fig. 5. Differences in the spectra can be explained in terms of surface charge, overall cell size and morphology, the number of charged groups in the cell wall, membrane conductivity and morphology, and the presence and internal conductivity of membrane-bound vesicles. The DEP spectra shown in Fig. 5 were obtained by direct observations of the dielectrophoretic effect. An alternative, and sometimes more rapid and accurate, method is to derive predicted DEP responses from electrorotation measurements on the bioparticles[5,17].

The cell wall, if present, consists mainly of complex charged polysaccharides and acts rather like an ion-exchange resin. Different species of bacteria are known to have large differences in their cell-wall properties, which gives them large differences in their effective particle conductivity (Table 1). By adjusting the medium conductivity, this can be exploited in the selective separation of bacteria from mixtures of different species[18].

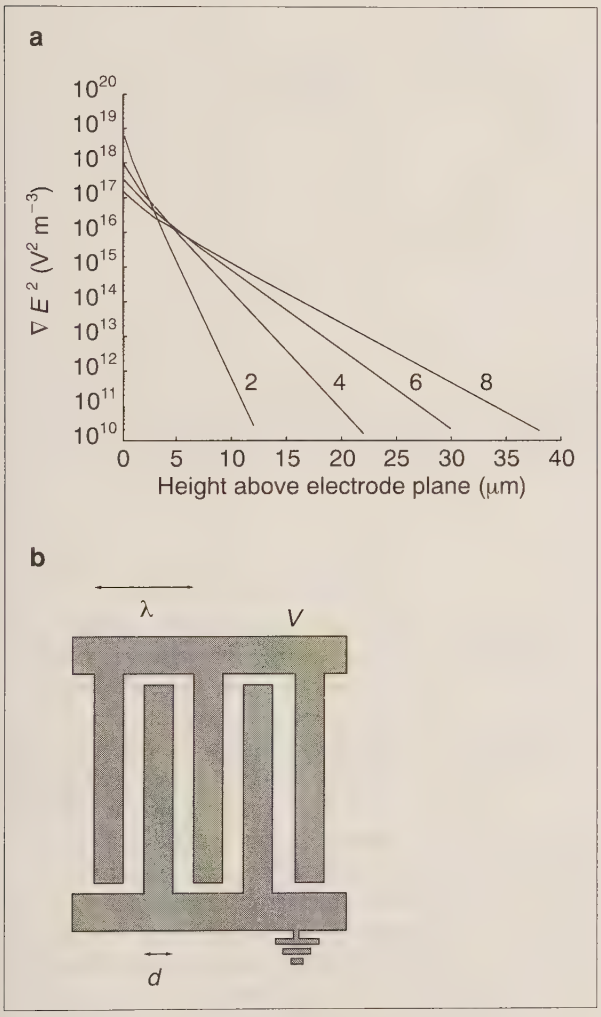

Figure 4

Variation of ∇E^2 with height (**a**) above a periodic array of interdigitated bar electrodes (**b**), for different electrode widths d (μm) and an applied voltage of 5 V rms. The factor ∇E^2 decays exponentially at a rate inversely proportional to the periodic length ($\lambda = 4d$) of the electrode array, while the maximum value of ∇E^2 is proportional to λ^{-3}.

Applications of DEP in biotechnology

Although one of the main uses of DEP in biotechnology to date has been in the alignment of cells prior to electrofusion[19], there is an increasing interest in applying it for the selective trapping, manipulation and separation of bioparticles. This interest stems partly from the ability to work at the single-cell or -organism level. While other techniques, for example optical tweezers and ultrasonic particle manipulation, can also be used to trap and isolate single particles, DEP-based systems have added advantages, including the simplicity of instrumentation and the ability to induce both positive and negative forces. The collection of cells at electrodes by positive DEP was demonstrated in the earliest work using macroelectrodes[2], but aggregation and trapping in regions away from electrode surfaces requires negative DEP and the development of microelectrodes, either in the form of 2-D structures[20,21] or so-called 3-D field cages[22,23]. One possible use of trapping includes the formation of aggregates and subsequent crosslinking or encapsulation, for example, for the production of artificial organs such as islets of Langerhans.

An early example of a DEP system for single-cell manipulation is the fluid integrated circuit proposed by Masuda *et al.*[7,24] These workers also introduced the use of travelling electric waves for the translational manipulation of particles along bar-shaped electrode arrays[25,26]. Particles such as erythrocytes were manipulated by this means, but because relatively low-frequency electric fields were used, electrophoretic rather than DEP forces were dominant. The technique was later extended to higher frequencies, at which DEP forces have the strongest effect on the motions of particles[27–35]. By employing multilayer fabrication technology, travelling-wave devices can be constructed so that only four main electrical connections are required to energize one or more electrode arrays of any length[30–33]. Linear travelling-field tracks are normally used, but they can also readily take the form of spiral[30,34], circular or forked (i.e. junction) geometries for incorporation into laboratory-on-a-chip devices[9]. Travelling-wave devices have been used to separate viable from unviable yeast cells[32], and white blood cells from erythrocytes[35].

If travelling fields are not used, then to achieve the spatial separation of particles, an additional force is needed to move the particles away from the local energy-potential minima. While in principle any force can be used, hydrodynamic forces, generated by a liquid flowing over the electrodes, are of particular interest. Because the electric fields are largely confined to areas close to the electrodes, and both particles and fluid flow are largely confined to narrow channels, such flow is mainly laminar. Many separations make use of periodically repeating electrode structures, usually of the interdigitated type, with or without castellations. In typical separations, particles are brought into the chamber by fluid flow and the particles attracted to the electrodes by a dielectrophoretic or gravitational force. By adjusting the

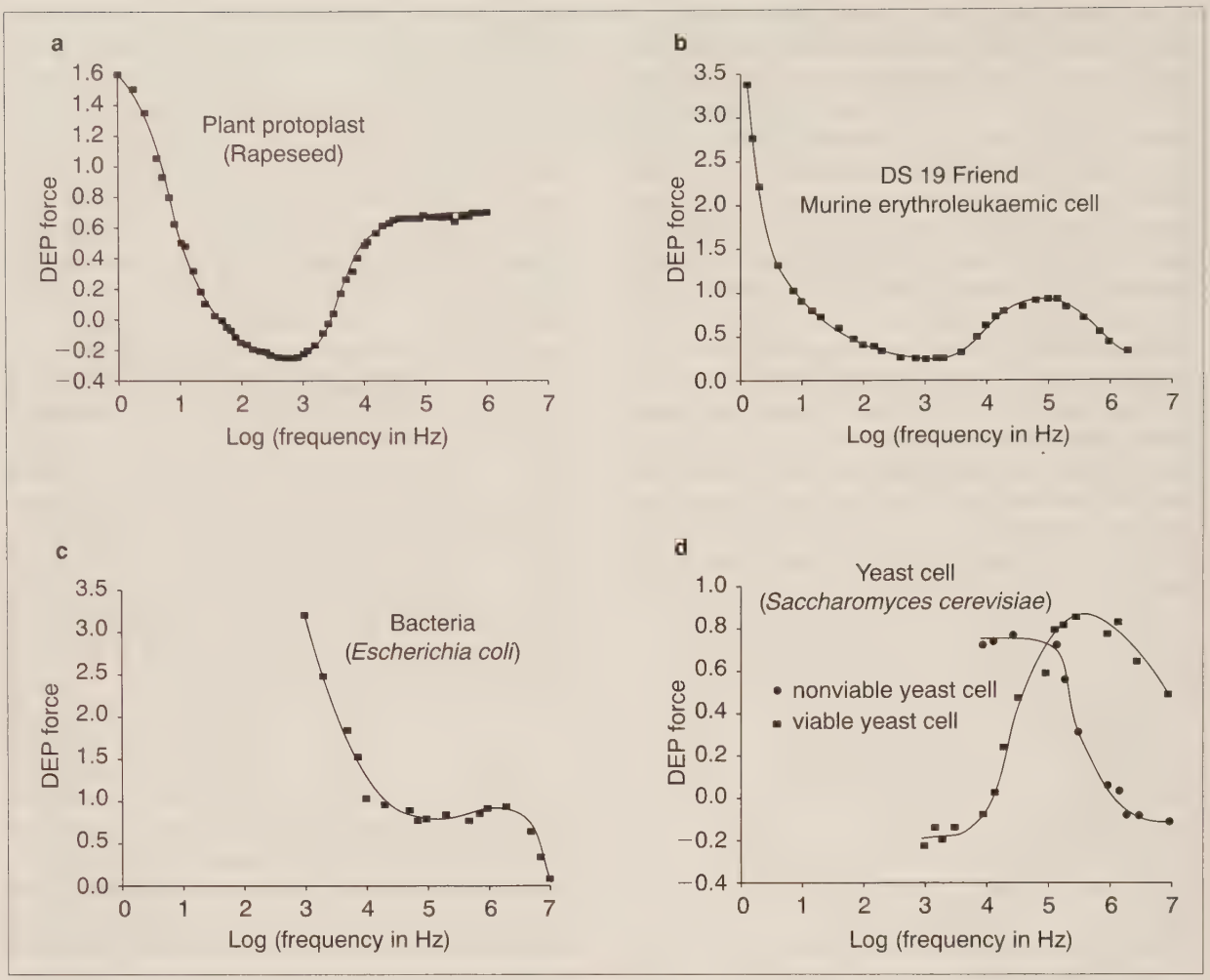

Figure 5

Dielectrophoretic spectra exhibited by different cell types when suspended in media of similar conductivity (3 ± 2 mS m^{-1}). (**a**) Rapeseed protoplasts[14], (**b**) DS19 Friend murine erythroleukaemic cells[15], (**c**) bacteria (*E. coli*)[16] and (**d**) yeast cells (*Saccharomyces cerevisiae*)[17]. The DEP forces are given in arbitrary units, and the important features of the spectra to note are the frequency ranges at which the DEP force changes polarity or varies rapidly with frequency.

conditions in the chamber, such as the frequency of the applied field or the conductivity of the suspending medium, separation is achieved by the selective release of certain particle types while other types are retained. By this means, batchwise separations have been achieved for viable and unviable yeast[17,36], several types of bacteria[16,18], leukaemia and breast-cancer cells from blood[37,38], and the concentration of $CD34^+$ cells from peripheral-stem-cell harvests[39]; continuous separation can also be achieved[40]. Apart from cells, biomacromolecules such as DNA[41] and proteins[42] have shown themselves amenable to DEP manipulation, as have viruses[43].

One promising line of development has followed the suggestion that DEP can be combined with the family of techniques known as field-flow fractionation[44]. In FFF, particles are injected into a carrier flow that passes through the separation chamber, with an external separating force being applied perpendicular to the flow. Through different factors, such as diffusion and steric, hydrodynamic, dielectric and other effects, or a combination thereof, particles with different properties attain different positions away from the chamber wall[44]. Particles that move further away from the wall reach higher positions in the parabolic velocity profile of the liquid flowing through the chamber and will be eluted from the chamber at a faster rate. Washizu *et al.*[42] were able to separate different sizes of plasmid DNA using positive DEP as the external force field with FFF, while Markx *et al.*[12] have investigated the use of negative DEP. By using negative DEP to levitate particles above the chamber wall into streams of the flowing liquid with different velocities, separation can be achieved in a similar way as hyperlayer-FFF[44]. This methodology has a number of advantages, including the ability to use high conductivities in the suspending medium and reduction of the adhesion of particles to the chamber walls. Also, because the DEP and gravitational forces have a similar dependence on the volume of the particle, particles with the same electrical properties and density but different sizes are levitated to approximately the same height above the electrodes, and separation is independent of particle size[12].

Do the electric fields cause cell damage?

As depicted in Fig. 1, when a cell is exposed to an electric field, it becomes electrically polarized. At low frequencies, the cell interior is shielded from the applied field by the effective capacitance of the cytoplasmic membrane, and as a result the membrane sustains the full electric potential applied to the cell[3,13]. The maximum value of the induced membrane potential occurs at the two 'poles' of the cell, in a radial direction parallel to the field, and has a value of about 1.5 rE. If this induced membrane potential exceeds a value of around 1 V, the electrical stress on the membrane can be sufficient to facilitate the useful effects of electroporation and electrofusion of cells[19]. For cells with a radius of around 2.5 to 5 μm, the applied field required to produce such effects is $1–3 \times 10^5$ V m^{-1}. As already described, a value for the factor ∇E^2 of around 10^{13} V^2 m^{-3} is required to produce a significant DEP force, and from Fig. 2, it can be seen that this can be achieved with an applied field of less than 10^4 V m^{-1}. The dielectrophoretic manipulation of cells should therefore not lead to any irreversible damage to the cells, and this has been confirmed in practice[36–39,45].

In experiments to demonstrate the DEP separation of viable and unviable yeast, the subsequent viability of the cells was checked by staining with methylene blue and plate counts[36]. The viabilities of erythrocytes dielectrophoretically separated from leukaemia cells have also been confirmed using trypan blue dye[37], and CD34+ cells have been successfully cultured following DEP separation[39]. Fuhr *et al.* have also demonstrated that fibroblasts can be successfully cultivated, without significant change in their viability, motility, anchorage or cell-cycle time, when exposed continuously over a period of three days to the fields generated by the types of microelectrodes used in DEP[45]. Bacteria are protected by strong cell walls, and from our own experience[16] no irreversible damage has been observed for a number of Gram-positive and -negative bacteria following exposure to DEP forces.

Table 1. The effective particle conductivities of a selection of bacteria are shown below. Based on such data, mixtures of bacteria can be separated by suitable adjustment of the suspending medium conductivity[16,18]

Gram-positive Species	σ_p (mS m^{-1})	Gram-negative Species	σ_p (mS m^{-1})
Micrococcus luteus	156±12	*Acinetobacter calcoaceticus*	< 2
Pediococcus damnosus	102±7	*Erwinia carotovora*	2±1
Bacillus subtilis	94±10	*Pseudomonas putida*	20±1
Bacillus megaterium	89±4	*Escherichia coli*	41±3
Lactococcus lactis	42±5	*Agrobacterium tumefaciens*	45±5
Enterococcus faecalis	23±2	*Klebsiella rubiacearum*	51±10
Lactobacillus brevis	15±2	*Rhodobacter sphaeroides*	97±5

For frequencies up to 100 kHz, the polarizability parameter α in Eqn 1 is mainly determined by the conductivities of the bioparticle (σ_p) and the suspending medium[16].

Concluding remarks

Following the introduction of microelectrode technology, dielectrophoresis has become a promising tool for the selective manipulation and separation of cells, bacteria and other microorganisms, as well as viruses and possibly biomacromolecules such as DNA and proteins. The method is noninvasive, does not require the use of antibodies or other labelling (although dielectric labelling to increase specificity can be an option) and can be employed at either the single-cell or multicell (more than 10^3 cells sec^{-1}) level. Applications in many areas can be envisaged. These include the monitoring and purification of cell cultures and the detection and removal of toxic pathogens from water, food and biological fluids. The isolation or enrichment of stem cells in bone marrow or peripheral blood, and of cell or organism subpopulations in heterogeneous samples is also possible. The technology can take the form of chromatographic chambers that employ flowing liquid, as in FFF, or use travelling electric fields to move the bioparticles selectively. Incorporation into 'laboratory-on-a-chip' devices is also a promising development[9].

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Membrane-protein engineering

Ismael Mingarro, Gunnar von Heijne and Paul Whitley

Membrane proteins perform many of the essential functions required for life. They are often the targets of medicinal drugs and have many potential uses in biotechnological processes. Therefore our ability to understand them and manipulate their functions is both important and necessary to enable protein engineers to create 'designer' membrane proteins (that is, proteins designed to have desired properties).

One of the main aims of membrane-protein engineering, as with protein engineering in general, is to be able to design and construct proteins with desired functions. However, before this can be successfully achieved, a sound knowledge of the basic properties of proteins, such as the rules governing their three-dimensional (3D) structure and their mechanisms of action are needed.

Very few structures of membrane proteins have been solved at high resolution, and thus little is known about the finer details of their function. Because of this lack of information, *de novo* design of membrane proteins is in its infancy and so far has been limited to rather simple motifs. The engineering of natural membrane proteins is, however, a very active research area and has provided a great deal of indirect structural information, as well as insights into the functioning, assembly and targeting of membrane proteins to their final destination within cells. This article will give a broad overview of some of the main recent applications of membrane-protein engineering.

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Structure and function

The fact that integral membrane proteins are, by definition, at least partially buried in the lipid bilayer appears to limit the types of structures that they can adopt, due to the physicochemical constraints that the hydrophobic environment imposes. So far, the protein structures that have been seen in membranes are the α-helices (20–25 amino acid residues in length), composed mainly of hydrophobic amino acids flanked by clusters of polar residues, and the β-barrels, in which hydrophobic residues face outward, towards the lipid bilayer.

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PII: S0167-7799(97)01101-3

The α-helix-type proteins are the most abundant and can be made up of a single helix or of multiple helices packed together in bundles. The orientation (topology) of helices in the membrane of many membrane systems (such as the bacterial inner membrane, the plasma membrane of eukaryotic cells, the inner membane of mitochondria and the thylakoid membrane of chloroplasts) is determined primarily by the distribution of positively charged amino acids flanking the transmembrane region according to the 'positive-inside' rule[1]. By altering the distribution of the positively charged amino acids in loops between transmembrane segments, a protein's topology in the membrane can be altered[2]. Such topological engineering has been applied to a number of membrane proteins in different membrane systems[3]. Membrane proteins made up of one, two and four artificial transmembrane segments, consisting of only the amino acids leucine and alanine, have been designed following the positive-inside rule and shown experimentally to have the desired unique topologies in the bacterial inner membrane[4] (Fig. 1).

The principle of the positive-inside rule has been applied to help overcome the problems involved in the overexpression of a mammalian olfactory receptor in *Escherichia coli*[5]. The wild-type receptor has a toxic effect on *E. coli*, whereas, by simply engineering positively charged residues into the first cytoplasmic loop, the protein can be overexpressed and purified, probably by preventing an inverted topology that affects insertion of the protein and blocks the translocation machinery.

Negatively charged amino acids also appear to play an active role in topology determination in *E. coli*, probably because of an electrophoretic effect caused by the potential across the inner membrane, which is positive at the periplasmic side[6] (Fig. 1).

Although there are good computational methods for predicting transmembrane helices from amino acid sequences based on hydrophobicity[7], it is not always clear which residues define the boundaries between the membrane and the aqueous environment. An experimental approach based on the observation that an engineered acceptor site for N-linked glycosylation can only be glycosylated in the lumen of the endoplasmic reticulum (ER) when it is situated more than 12 residues away from the membrane boundary residue may be of general use in determining the ends of transmembrane helices using engineered glycosylation sites as a 'molecular ruler'[7].

Cysteine: the most wanted

Transmembrane helix–helix interactions play a major role in the assembly of membrane proteins into their 3D structures, both individually and in complexes[8]. Many genetic-engineering approaches have been used to obtain information on how transmembrane helices pack together in bundles; one that may be applicable to a wide variety of membrane proteins is to look for the formation of disulphide bridges between pairs of cysteine residues engineered into transmembrane helices. As a disulphide bond will only form when the cysteines are in close proximity to each other[9], analysis of the ability of a number of cysteine pairs to form disulphide bonds can be used to determine how helices are oriented with respect to each other, as has been shown in a number of proteins, including M13 coat protein[10], bacterial leader peptidase[11] and bacterial chemoreceptors[12,13].

The engineering of pairs of cysteine residues to form disulphide bonds is not only used to probe protein structure but also has been used for other purposes such as the overexpression of a toxic protein. The engineering of two cysteine residues between helices 5 and

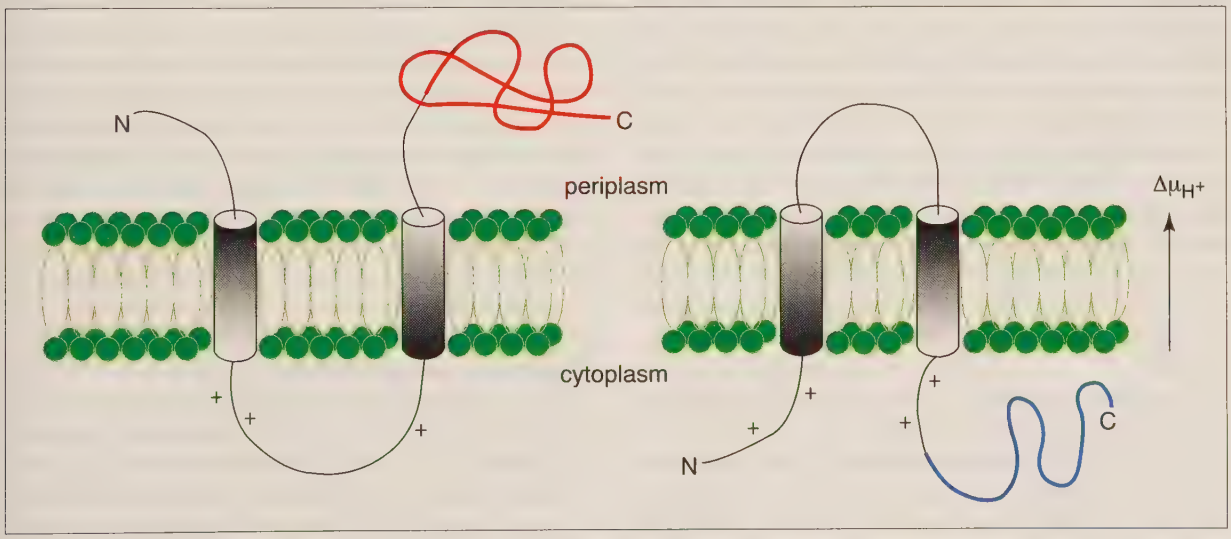

Figure 1

Topological engineering: the effect of the distribution of positively charged amino acid residues on the orientation of a hypothetical *Escherichia coli* inner-membrane protein with two transmembrane segments. The potential across the membrane, $\Delta\mu_{H^+}$ (acidic and positive in the periplasm), influences the topological effects of charged residues. Fusions (thick line) to either periplasmic (red) or cytoplasmic (blue) regions of the membrane protein are useful for determining the topology of the membrane protein.

6 of the colicin-A pore-forming domain, which is normally cytotoxic to *E. coli*, elegantly overcomes the toxicity problem, probably by preventing membrane insertion of these two helices[14].

The special reactivity of cysteyl side chains can also be used to target chemical modifications, and this has been exploited in a number of different ways. The covalent attachment of small probes, such as fluorescent, radioactive or spin-labeled molecules, and the pattern of labeling or the properties of the attached label can be used to provide information about the environment of the attached, modified amino acid and hence clues as to the structure of the membrane protein.

E. coli lactose permease has had each of its 417 residues individually changed to cysteine, and is the most complete example of cysteine-scanning mutagenesis to date. This library of cysteine mutants has been analysed for biological function and by a number of biophysical techniques aimed at obtaining structural information[15].

Beyond cysteine

An extensive analysis of the glycophorin-A (GpA) homodimer has been performed using saturation mutagenesis, in which the effects on dimerisation of randomly introducing mutations into the transmembrane segment were studied[16]. From the data obtained in this study, a detailed model of the helix interactions in the GpA dimer could be constructed. The power of this saturation-mutagenesis study has recently been confirmed by the determination of the NMR structure of the glycophorin transmembrane-helix interface[17].

Although saturation mutagenesis is a very powerful technique, it requires the analysis of a huge number of mutants. Recently, an insertion (rather than replacement) mutagenesis approach has been used rapidly to identify the most critical segments involved in GpA helix packing[18]. The rationale for this approach is that the insertion of an amino acid into an α helix will displace the residues on the N-terminal side of the insertion by 100° relative to those on the C-terminal side, thus disrupting helix-packing interfaces (Fig. 2). As insertion mutagenesis is more disruptive than replacement mutagenesis, it may be useful in general as an initial, rapid screen for structurally and functionally important segments of transmembrane helices, which could then be complemented with a more extensive saturation-mutagenesis study of these important regions.

Tryptophan is a bulky hydrophobic residue that has been postulated to be tolerated as a replacement for amino acids facing the lipid bilayer but not for amino acids in the protein interior, where it would be disruptive. Scanning transmembrane segments with tryptophan has been applied to flagellar-motor components, the MotA and MotB proteins of *E. coli*, in which it was shown that the replacements that did not disrupt function would be predicted to lie on one face of a helix, the one facing the lipid[19,20].

An interesting feature of transmembrane helices is their relatively high proline content compared with α-helices of globular proteins. Proline disrupts α-helices and introduces a kink of approximately 26°, which, in transmembrane helices, is thought to create a convex face adjacent to other helices and a concave side facing the lipid bilayer[21]. The function of transmembrane prolines has recently been examined in both the lutropin–choriogonadotropin receptor, which has five transmembrane prolines[22], and the cystic-fibrosis transmembrane-conductance regulator, which has two[23]. In both cases, replacement of the individual prolines alters the function in different ways, indicating that, although they are important, there is no universal role for prolines in transmembrane helices.

It is possible to place specialised, artificial amino acids into membrane proteins by suppressing nonsense codons with chemically misacylated synthetic tRNA specifically designed for the incorporation of artificial amino acids. In *Xenopus* oocytes, the tachykinin neurokinin-2 (NK-2) receptor (a G-protein-coupled receptor) has been labeled with fluorescent amino acids at specific positions[24]. Intramolecular distances within the protein could be determined by measuring fluorescence-resonance energy transfer (FRET) between the artificial amino acid and a fluorescently labeled NK-2-antagonist molecule. This method of suppression mutagenesis and FRET could, in principle, be applied to many proteins and be of general use in the analysis of spatial relationships in integral membrane proteins, such as receptors or channels.

There are many examples of mutational analysis of membrane proteins that have been aimed at identifying amino acids or regions responsible for a particular function of the protein, such as ligand binding (for receptors) or ion specificity and gating (for ion channels). Interpretation of the results from these types of experiments is often difficult, as a mutation that knocks out a function can do so for a number of reasons, ranging from being specific for that function to destabilising the protein as a whole. A variation on this theme is to create a mutation that knocks out a function and then look for a second (suppressor) mutation that will restore function. These types of experiment have been carried out with a number of proteins and can provide both stuctural and functional information. The role of charged residues and ion pairs in transmembrane helices have been particularly targeted by this approach[25].

Chimeras: more than a dream

The construction of chimeric proteins is another way of determining the regions of proteins responsible for particular functions. Related proteins with slightly different functions, such as a different ligand specificity or signaling pathway, can be engineered to create chimeras that have the properties of one or other of the parent molecules or, in some cases, hybrid properties. The analysis of chimeric proteins can often be useful in assigning functions to particular regions of proteins that would be difficult using conventional

mutagenesis. A recent study with chimeras of retinal and olfactory cyclic-nucleotide-gated channels suggested that the channels possessed a cyclic allosteric mode of activation, and that the N-termini of these proteins, distinct from the pore- and ligand-binding motifs, are involved in the allosteric gating transition[26].

Chimeras between membrane proteins and alkaline phosphatase have been extremely useful in experimentally determining membrane-protein topology in *E. coli*. Fusions with periplasmic loops of membrane proteins result in enzymatically active and protease resistant alkaline phosphatase, whereas cytoplasmic fusions are inactive and protease sensitive (see Fig. 1). Fusions to β-galactosidase show the reciprocal behavior to the alkaline-phosphatase fusions: periplasmic fusion sites yield hybrid proteins with reduced specific activitites, which are usually highly toxic if expressed at high levels, whereas cytoplasmic fusion sites yield hybrid proteins with high specific activities. The combined use of alkaline-phosphatase and β-galactosidase fusions thus provides strong experimental evidences that can verify the topological models[27].

An extreme example of a chimera is glycophorin A (mentioned above), in which a transmembrane helix was taken out of context and fused to an unrelated soluble protein such as staphylococcal nuclease[28]. Helix–helix interactions could then be followed by looking at transmembrane-induced oligomerisation of the nuclease in the presence of detergent. The dimerisation motif of GpA, which comprises seven amino acid residues, can also be taken out of context and grafted into other helices, such as a polyleucine stretch or the transmembrane fragment of the *ToxR* transcription activator from *Vibrio cholerae*, and driving dimerisation in SDS[29] or in a natural membrane, respectively [30].

Assembly and membrane-protein trafficking

Engineered membrane proteins have contributed greatly to our understanding of how membrane proteins insert into membranes. The ability to incorporate UV-light-inducible cross linkers into membrane proteins at specific positions *in vitro* using modified peptidyl-tRNA molecules, in particular, has provided much data on the machinery involved in membrane-protein assembly into the ER (the translocon). Photocrosslinking data show that integrating transmembrane segments are likely to follow an ordered multistep pathway, because they encounter at least three proteinaceous environments in the translocon during assembly before finally ending up in a lipid-exposed location. Furthermore, many of the protein components of the translocon, such as Sec61α and translocating-chain-associated membrane protein (TRAM), have been identified with the aid of photocrosslinking[31,32]. (For a fuller treatment of the membrane-protein assembly problem, see Ref. 3.)

The sorting of proteins to different cellular compartments (especially in eukaryotes) is another important area of research that has been greatly aided by protein engineering. Many of the signals for targeting membrane

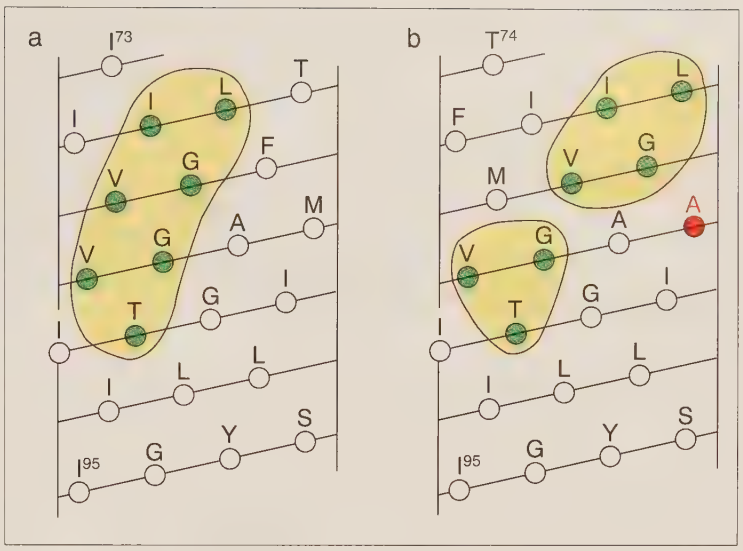

Figure 2

(**a**) The seven critical residues (green) in the glycophorin-A transmembrane helix, mapped onto a helical net diagram; the N-terminus is at the top. (**b**) The disruption of the interface motif (yellow area) by an alanine insertion (red) between positions 81 and 82.

proteins to different organelles have been elucidated using site-directed mutagenesis and the construction of chimeric proteins, as removal or addition of specific sequences in membrane proteins can often lead to the proteins being targeted to different subcellular locations. Signals containing tyrosine, such as YXXO (where Y = tyrosine, X = any amino acid and O is an amino acid with a large hydrophobic group), situated in the cytoplasmic domains of membrane proteins are involved in both internalisation from the cell surface and also localisation of proteins to the trans-Golgi network. These signals bind to clathrin-associated proteins, which suggests that they are sorted by clathrin-dependent sorting mechanisms. Other linear sequences, such as KKXX or XXRR (where K = lysine and R = arginine), are necessary for the retrograde movement of membrane proteins from the Golgi apparatus to the ER via coatamer-mediated mechanisms. An example of such a sequence is the six amino acid carboxy-terminal sequence of the resident ER membrane protein E19 (DEKKMP), which, when encoded by adenovirus and transplanted onto the T-cell-surface glycoproteins CD4 or CD8, has been shown to be necessary and sufficient to target these proteins to the ER[33,34].

Designer membrane proteins

Peptide mimics of channel-forming proteins such as the acetylcholine receptor have contributed to our understanding of the mechanism of transmembrane ion conductance[35]; recently, a small globular protein (69 amino acids in length) containing a buried hydrophobic helix has been synthesised that can insert into lipid bilayers, possibly in a manner similar to colicins, to form transmembrane pores[36]. These relatively simple peptides and small proteins may be far from perfect, but do provide a framework onto which

Table 1. A summary of some of the applications of membrane-protein engineering

Goal	Examples	Comments	Refs
Define topology	Leader peptidase, artificial transmembrane segments	The distribution of positive charges drives the protein topology.	2, 4
	Coat protein of the *Pseudomonas aeruginosa* phage Pf3	Active role for negatively charged amino acid residues.	6
Protein overexpression	Olfactory receptor	The addition of three positively charged residues in the first cytoplasmic loop allows protein overexpression.	5
	Colicin A	Formation of a disulphide bond prevents the cytotoxic activity of the protein and its overexpression.	14
Determine the ends of a transmembrane segment	Leader peptidase, polyleucine transmembrane segments	Glycosylation mapping at the lumenal side of the endoplasmic reticulum.	7
Define 3D structure	M13 coat protein, leader peptidase, Trg receptor, lactose permease	Cysteine scanning.	10–13, 15
	Glycophorin A	Random mutagenesis.	16
	Glycophorin A	Alanine-insertion scanning.	18
	MotA and MotB proteins	Tryptophan scanning.	19, 20
	Tachykinin neurokinin-2	Incorporation of artificial amino acids.	24
	Tachykinin neurokinin-1, lactose permease	Metal-binding by point mutation (histidine).	39, 40
Protein function	Lutropin–choriogonadotropin receptor, cystic-fibrosis transmembrane-conductance regulator	Native proline substitution.	22, 23
Switchable pores	K^+ channel	Point mutation (cysteine).	37
	Staphylococcal α-toxin	Replacement of five native residues by five histidines.	38
Crystallisation	Lactose permease	A soluble 'carrier' protein (cytochrome b_{562}) was fused in order to increase the polar surface area.	50
	Escherichia coli quinol oxidase periplasmic fragment	Periplasmic fragment was overexpressed and purified.	51

more complex functions and regulatory features can be built.

In general, *de novo* protein designs are aimed at creating sequences capable of forming a given 3D structure (and possibly also having a specific function). In membrane proteins, the introduction of metal-binding sites raises the possibility of using such engineered sites as chemical switches for controlling the function of transmembrane channels, but also as a general tool in the structural and functional characterisation of helix–helix interactions. For example, a K^+ channel has been engineered to incorporate a metal-controlled switch by introducing a cysteine residue into the extracellular region of the channel, resulting in Zn^+- and Cd^+-dependent gating effects[37]. More recently, an extension of a previous study on staphylococcal α toxin has shown that the replacement of residues 130 to 134 of the native toxin by five consecutive histidines produces a 2 nm pore equipped with a metal-activated switch that reversibly permeabilises the plasma membrane to small molecules (≈1000 Da) in living cells[38]. This ability to control the transmembrane flux of molecules and yet maintain cell viability could have numerous biotechnological applications.

The engineering of a metal-ion-binding site into the human tachykinin NK-1 receptor by the systematic introduction of histidyl residues at the antagonist-binding site gradually converts it into a high-affinity metal-ion-binding site without affecting agonist binding, allowing the molecular model of this receptor to be tested[39]. An engineered metal-binding site in lactose permease has also been used to confirm the proximity of two transmembrane helices; by using electron paramagnetic resonance, it was shown that just one point mutation (E269H) causes the permease to bind Mn^{2+} with a stoichiometry of unity, whereas the wild-type permease exhibits no binding[40].

Attempts have been made to design and synthesise soluble versions of membrane proteins. For example, soluble 'maquettes' of transmembrane helix bundles have been synthesised that are based on the cytochrome *b* subunit of the cytochrome bc_1 complex, and in which the lipid-exposed residues have been changed to confer amphiphilic character and water solubility on the α helices. The resultant peptide

assemblies are not only stabilised by addition of ferric heme, but also demonstrate negative cooperativity in heme binding[41].

Membrane engineering

This article has mainly focused on the manipulation of membrane proteins. However, it must be noted that the phospholipids in the membrane have a strong influence in maintaining and determining the final structure of such membrane proteins as transporters (e.g. lactose permease) in functional conformations[42], and are also involved in the process of protein translocation across the plasma membrane of *E. coli*[43]. It is now possible, using specific strains of *E. coli*, to engineer membranes so that they have different phospholipid contents than normal *E. coli* membranes, thus providing the means to investigate the *in vivo* role of lipids on many different aspects of membrane function[44]. These *E. coli* strains include: strains lacking cardiolipin[45], strains in which phosphatidylglycerol levels can be varied[46], strains lacking phosphatidylethanolamine[47], strains containing phosphatidylcholine[48] and strains in which phosphatidylinositol can be introduced[49].

Conclusion and future prospects

A short review such as this only scratches the surface of the field of membrane-protein engineering, but should provide the reader with an idea of the diversity of its applications (Table 1). Understanding integral membrane proteins has been, and continues to be, difficult, owing to the relative paucity of high-resolution structural data. However, the development of genetic engineering, combined with genetics, biophysics and biochemistry, is providing useful tools for filling in some of the many gaps in our knowledge. If techniques for studying membrane proteins advance in the next decade as they have in the past decade, then the protein engineers' goal of being able to design a membrane protein with desired properties may well be within reach early in the next millennium.

Acknowledgements

Work performed in the authors' laboratory was supported by an EMBO postdoctoral fellowship to I. Mingarro and grants from the Swedish Technical Sciences Research Council, the Swedish Natural Sciences Research Council, the Swedish Cancer Foundation and the Göran Gustafsson Foundation to G. von Heijne.

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Changing animals

Transgenic Animals: Generation and Use

edited by Louis Marie Houdebine, Harwood Academic Publishers, 1997. UK£59.00 pbk (xi + 576 pages) ISBN 9 05702 069 6

Techniques permitting the modification of the whole animal genome were developed over 15 years ago. After an initial lag phase during which a few key experiments demonstrated its potential, this area of science expanded rapidly, and the use of transgenic animals has continued to grow. The high frequency of relevant publications from a wide range of disciplines is testament to the benefits of this approach. Over the years, numerous reviews and a number of textbooks have dealt with the subject, including the excellent technical manual *Manipulating the Mouse Embryo*. Another text in this area would seem to be superfluous, and yet this book manages to be informative, interesting and, above all, comprehensive. Divided into five parts and 83 (albeit short) chapters, with a total of 161 contributors, this text covers every aspect of gene transfer in whole animals. A range of mammalian species is included, as well as sections on birds, fish and the standard invertebrate models. Equally diverse is the coverage of different methods of introducing DNA; discussions on pronuclear microinjection, retroviral vectors and embryonic stem cells are to be expected, but there are also chapters on adenovirus vectors, ballistics and primordial germ cells.

All too frequently, experiments are blighted by poor or inappropriate levels of transgene expression. Our understanding of the mechanisms controlling gene regulation remains incomplete, and tackling these problems will be crucial to improving and expanding the utility of transgenic technology. This book devotes considerable space to approaches designed to overcome position effects and obtain faithful expression of the introduced genetic material. Included in the text are sections on YACS, BACS, transgene insulators, gene targeting and inducible systems.

Any review of transgenic applications is problematical, owing to the multitude of different disciplines that have successfully exploited this technology, but this area is handled well and includes the general approaches that can be used to investigate biological systems, as well as more-specific sections on current and developing uses for transgenic models and technologies.

The presentation style – a large number of short chapters on specific topics – leads to a small amount of overlap, but allows relevant information to be found with ease. This multiauthored approach results in a varied layout, although this in itself does not detract from the whole. In general, the standard is high, but inevitably some chapters are more informative than others and, in a small number of cases, the authors have dealt with their subject matter a little too superficially. A few notable names are missing from the list of contributors, but omissions of substance are rare; the most obvious of these is the recent work of I. Wilmut and co-workers, which is referred to only in passing. However, these are very minor criticisms, the main strength of this book being the diversity of topics included and the very large number of contributors, which has ensured well-rounded coverage of the subject. This book is recommended to anyone who is responsible for the production of transgenic animals or has a general interest in the subject.

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First find your protein

Recombinant Protein Protocols: Detection and Isolation

edited by R. S. Tuan, Humana Press, 1997. US$74.50 (xx + 448 pages) ISBN 0 896 03400 3

Recombinant Protein Protocols is one of the latest offerings from the *Methods in Molecular Biology* series. It aims to fulfil 'the need for a laboratory manual on the detection and isolation of recombinantly expressed genes that covers the background information and the practical laboratory recipes for these analyses.' The editor must be congratulated for managing to maintain the strict format that flows throughout the book; it helps to guide the reader through each technique quite clearly. For those not familiar with a particular protocol, the introductions to each chapter, are generally successful in giving the relevant background information, but there are some that are poorly described, and leave you to wonder about the application and relevance of the technique. Each protocol is also followed by a section of notes; the authors have been meticulous in presenting both the reasons why certain steps are required and useful alternatives that can be employed in specific experimental circumstances. Overall, I feel that the book provides the researcher with enough information to make a considered judgement about the usefulness of a technique, and allows the experiment to be performed with a certain degree of confidence.

The greatest weakness of this compilation, however, can be predicted by its title. In attempting to mirror such a large field, some readers may find that many chapters are of little practical relevance. The book will be most useful to those who are working in the fields of developmental biology and gene therapy, but for the remainder, it can still provide a valuable insight

into techniques outside one's immediate area of expertise.

The book is divided into three sections, under the titles of 'Detection and isolation of expressed proteins', 'Detection of recombinant gene expression *in situ*', and 'Applications of recombinant gene expression'. Within the first section, there is a series of methods covering the current state of reporter-gene technology, with a slight bias towards chemiluminescent detection. There are detailed protocols for the bacterial expression and purification of recombinant proteins fused to maltose-binding protein, staphylococcal protein A, streptavidin and a histidine-tagged GAL4 DNA-binding domain. Two chapters describe methods for the identification and analysis of interacting proteins using yeast genetics – the now-traditional GAL4-based system – and also an alternative using the bacterial LexA protein. It would have been more informative had protocols describing other approaches to the problem, such as phage display, been discussed. There are also some rather specialized cell-biology techniques, and a well-written chapter describing the immunopurification of proteins on an immobilized support, with protocols ranging from hybridoma culturing to elution from a column.

The second section is probably the strongest, because it describes up-to-date protocols from the more tangible area of *in situ* hybridization and immunohistochemistry. Protocols are given for the analysis of RNA and protein expression, including dual detection, detection in tissue sections and whole-mount embryos. For those requiring extremely high sensitivity, the technique of *in situ* PCR is included for a number of tissue types. The section concludes with a review of the green-fluorescent-protein field, which is excellent for anybody wishing to use it as an expression tag.

The techniques described within the final section concentrate mainly on technologies currently being designed for use in the field of gene and cell therapy. These are very specific and contain protocols that are useful in very few laboratories. For most, it will serve only to give an insight into the current problems being encountered, together with examples of the diverse ways in which attempts are being made to overcome some of these hurdles.

Overall, I must reiterate that the book is very well written, and describes many current protocols in a clear format. However, if you are looking purely for protocols, then check out the contents page carefully; there may be books of more relevance on the shelf.

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Modelling enzyme reactions

Computer Modelling of Chemical Reactions in Enzymes and Solutions

by A. Warshel, Wiley Interscience, 1997. UK£39.95 pbk (xi + 236 pages)
ISBN 0 471 18440 3

In a relatively small number of pages, Arieh Warshel covers a considerable amount of territory in this extremely helpful guide to the theory of enzymic reactions. Bearing in mind that this can be a rather difficult subject for some, it is very well written and also fairly readily understandable, although a background in theoretical chemistry or quantum mechanics would be an advantage.

The introductory chapter deals with molecular orbital theory in a refreshingly lucid manner, carefully describing the way in which approximations to the Schrödinger equation can be applied to diatomic and polyatomic molecules. The book then leads into the coverage of reaction-rate theory for the gas phase and in solution, with molecular dynamics and free-energy-perturbation methods being introduced in Chapter 3. The application of molecular-mechanics and -dynamics simulations on macromolecules is explored in the following chapter, with theoretical aspects of enzyme kinetics introduced in Chapter 5; both these chapters include a consideration of solvent effects.

Specific enzymic systems are explored in the next three chapters, dealing with (in order) lysozyme, the serine proteases and selected metalloenzymes, including staphylococcal nuclease and carbonic anhydrase. These treatments all clearly illustrate the application of theoretical techniques and computer-modelling procedures to the understanding of enzyme reactions. The final chapter provides a more general outlook on key factors of importance in the majority of enzymes, such as conformational change, electrostatics, desolvation energy and entropic effects. As with the previous chapters, this demonstrates how computer-generated information based on theoretical considerations of enzyme catalysis can provide important insights into the way enzymes work.

This book will prove useful for those interested in applying theoretical methods to enzyme systems in order to gain a further understanding of enzyme-catalysed reactions at the molecular level. Now that we have the computational resources for handling extensive calculations on complex macromolecular systems, it is possible to obtain a detailed picture of how biological reactions are catalysed. Accordingly, a noteworthy inclusion to this text is the listing of a number of relevant computer programs, which enable some of the procedures described to be carried out on a PC. In addition, the subject matter is richly illustrated with diagrams and other clearly reproduced figures, some of which are in full colour. Consequently, this attractively-priced paperback edition of an important fundamental work in an area of growing interest should prove to be of great benefit to both students and researchers in the biological sciences.

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Transgenic plant technology is entering the era of metabolic engineering

Meeting report

Basic research discoveries in the 1980s related to the genetic transformation of crop species have led to the commercial introduction of transgenic crops in the 1990s. These new varieties have novel single-gene traits that are, in general, of value to the farmer (e.g. resistance to insects, crop-protection chemicals or viruses). In recent years, molecular biologists and biochemists have begun unraveling the molecular basis of the more-complex processes in plants. The emerging subdiscipline termed 'functional genomics' reflects a growing interest in identifying the genetic basis of developmental or metabolic pathways, and the control systems of complex cellular functions. It is anticipated that this research will have a profound impact on our basic understanding of plant science, and also lead to the creation of the next generation of improved crops. To review the current state-of-the-science, a Keystone Conference was recently held to discuss these issues*. 195 people attended the meeting, with representatives from 16 different countries and over one-third coming from outside the USA. The sessions were organised to highlight specific biosynthetic pathways or cellular processes that influence the genetic modifications of these pathways.

Plant-gene suppression and disruption

Suppression of gene expression in transgenic plants was referred to often in the meeting, either as an approach to crop improvement or as an unwanted side-effect of the presence of a transgene. Two presentations, from R. Kumar (Kimeragen Inc., Newtown, PA, USA) and D. Baulcombe (Sainsbury Laboratory, Norwich, UK) dealt with novel approaches to the specific suppression of gene expression that will have general utility as research tools and be at the cutting edge of crop improvement. R. Kumar described a targeted gene-disruption procedure referred to as '*in vivo* mutagenesis' in which a hybrid RNA–DNA oligonucleotide (a 'chimeroplast') anneals with homologous DNA *in vivo*. The bias in his presentation was towards gene therapy in animals but, in the subsequent discussion, there was optimism that the approach could be adapted for work with plants. D. Baulcombe described virus-induced gene silencing in plants infected with modified viruses carrying fragments of plant genes, apparently mediated by a signal that diffuses away from the infected parts of the plant. One implication of virus-induced gene silencing is that viral RNA is an activator of gene silencing and so genes or transgenes that are similar to either the viral genome or an insert may be silenced. This approach to gene silencing was consistently effective in many independent transgenic lines and, therefore, may have some advantages over antisense or cosuppression, which are normally only partially effective, variable between lines and unstable.

To better understand the transgenic factors influencing cosuppression, the laboratory of J. Mol (Free University, Amsterdam, The Netherlands) has analysed many lines of petunia that are cosuppressing pigment-biosynthetic genes. They concluded that the presence of inverted-repeat structures at the transgene locus favors cosuppression and that the transgene initiator of gene silencing need not be transcribed. J. Mol also presented a model to explain his view of the underlying mechanism, one of the novel features of which was the formation of cruciform DNA involving intrastrand base pairing of the inverted-repeat DNA. He suggested that these cruciform structures promoted DNA methylation and that the DNA methylation was somehow implicated in the interaction between the transgene locus and the endogene target of the silencing mechanism.

It has been widely thought that variation in chromatin structure at, or adjacent to, a transgene locus is a major factor in gene silencing and between-line variation in trangene expression. However, D. Ow (Plant Gene Expression Center, Albany, CA, USA) showed that independent, but otherwise identical, insertions at the same locus (using Cre–lox-mediated recombination at specific sites in the genome), were not all expressed at the same level. At one level, this result is a disappointment, as it had been hoped that site-directed transgene integration would provide a way of minimising between-line variation in transgene expression. However, at the scientific level, this is extremely interesting and unexpected – clearly there are factors affecting transgene expression that are currently beyond our comprehension.

Signal-transduction pathways for plant defense responses

The orchestration of induced metabolic events as plants respond in a genetically determined manner to pathogen invasion provides a model for understanding the coordinated transcriptional control of plant biosynthetic pathways. W-Y. Song (University of California, Davis, USA) summarised the characterisation of the rice Xa21 gene, which confers resistance to 31 isolates of the pathogenic bacterium *Xanthomonas oryzae* pathovar oryzae. The sequence of the predicted Xa21 protein, which carries both a leucine-rich repeat and a serine–threonine kinase motif (the latter shown to be active), suggests a role in cell-surface recognition of a pathogen ligand and subsequent activation of an intracellular defense response. The homolog Xa21D encodes a truncated version of Xa21 that lacks these domains owing to insertion of a transposable element (a common feature in the Xa21 gene family), but still conferred partial resistance (a phenomenon with interesting parallels to self-incompatibility in brassicas).

J. Jones (Sainsbury Laboratory) discussed the characterisation of the Cf-2, Cf-4, Cf-5 and Cf-9 genes and their homologs in tomato, which confer resistance to races of the fungus *Cladosporium fulvum* that express the corresponding avirulence genes Avr2, Avr4, Avr5 and Avr9, respectively. The sequence of the Cf genes predicts a predominantly extracytoplasmic glycoprotein with

*The *Metabolic Engineering in Transgenic Plants* conference, organised by the Keystone Symposia and sponsored by the Samuel Roberts Noble Foundation, was held at Copper Mountain, CO, USA, 12–16 April 1997.

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PII: S0167-7799(97)01115-3

numerous leucine-rich repeats and a short C-terminal membrane anchor. To identify immediate downstream signal-transduction events, J. Jones and co-workers developed a Cf-9 transgenic-tobacco suspension-culture system that responds to Avr9 by producing high levels of reactive oxygen species.

D. Klessig (Waksman Institute, Rutgers State University, Piscataway, NJ, USA) described his group's studies on perception and transmission of the salicylic acid signal, an important secondary signal in the defense pathway in some (e.g. tobacco – tobacco mosaic virus), but not other (e.g. tomato – *C. fulvum*), plant–pathogen systems. A number of potential components in the pathway between salicylic acid and induction of pathogenesis-related genes were identified.

C. Lamb (Salk Institute, La Jolla, CA, USA) focused on binding (activation) of the bean chalcone synthase (chs)15 promoter, one of the more extensively characterised promoters induced during defense responses to microbial pathogens. Related G- and H-box sequences in this promoter are involved in activation by pathogens and/or elicitors. A basic leucine-zipper G-/H-box-binding factor (G/HBF1) was cloned, whose binding is activated post-translationally by a Ca^{2+}-dependent protein kinase and potentially deactivated by a newly cloned phosphatase. A second G/HBF, termed KAP2, was also cloned following classical purification of the factor. Under certain circumstances, G/HBF1 and KAP2 can interact at the G box to form a new protein–DNA complex, suggesting the involvement of multiple DNA-binding proteins in the activation of this promoter. Lamb stressed the potential for engineering plant-metabolic pathways by transgenic expression of such regulatory proteins.

Genetic manipulation of natural-product pathways

The past five years have seen major advances in our understanding of the biosynthetic pathways leading to the major classes of natural plant products – the phenylpropanoids, isoprenoids and alkaloids. With the cloning of the genes encoding many of the important biosynthetic enzymes involved in these complex pathways, the genetic manipulation of end products, both quantitatively and qualitatively, is now feasible. R. Dixon (Noble Foundation, Ardmore, OK, USA) outlined some of the limitations to secondary-metabolite engineering, and discussed approaches that may help to overcome them. R. Croteau (Washington State University, Pullman, WA, USA) described his pioneering work on the cloning of the cyclase and P450-hydroxylase enzymes involved in the biosynthesis of monoterpenes, important compounds in the fragrance and flavor industries. This work has been facilitated by the ability to isolate the glands involved in secretion of these compounds, as a starting point for enzyme purification.

T. Kutchan (University of Munich, Munich, Germany) pointed out that over 12 000 different alkaloids have been characterised from plants, with 1800 members of the bis-benzylisoquinoline class. A major theme of her talk was the plasticity of enzyme-product specificity within the alkaloid biosynthetic pathways. For example, different products can be obtained from the same cytochrome P450 enzyme by coupling to cytochrome P450 reductases from different sources. She predicted a use for recombinant alkaloid biosynthetic enzymes in 'combinatorial biotechnology' for the production of novel pharmaceutical products.

R. Last (Boyce Thompson Institute, Ithaca, NY, USA) described the genetic approaches aimed at understanding ozone tolerance and antioxidant-biosynthetic pathways being carried out in *Arabidopsis*. An ozone-sensitive mutant, soz-1, which is also sensitive to other free-radical-generating stresses such as high UV-B and sulfur dioxide, has significantly reduced levels of ascorbic acid, and precursor-feeding experiments suggest that soz-1 is an ascorbate-biosynthetic mutant. Surprisingly, the biosynthetic pathway to ascorbate is not fully understood in plants. In addition to indicating the power of *Arabidopsis* genetics for dissecting complex physiological processes, this work indicates the value of genetic screens for the analysis of basic metabolic pathways.

Carbohydrate biosynthesis and regulation

L. Willmitzer (Max Planck Institute, Berlin, Germany) described studies of the genetic manipulation of the starch content and properties in potato. In an attempt to obtain new targets for starch modification, Willmitzer's group produced antibodies against the proteins associated with starch grains and used these as probes to obtain cDNA clones encoding novel starch-related proteins. Functional analysis by antisense down-regulation revealed that one gene, designated R1, controlled starch phosphate content. Reducing R1 expression led to decreased starch viscosity and less free sugars on cold storage, a good trait for varieties produced as frying potatoes.

Plant carbohydrates also play important roles during plant stress. H. Bohnert (University of Arizona, Tucson, AZ, USA) described the importance of sugar alcohols as compatible solutes in osmoprotection and the effects of overproduction of such compounds in transgenic plants. Surprisingly, their protective effects may result more from antioxidant activity than from the maintenance of osmotic balance. S. Smeekens (University of Utrecht, The Netherlands) discussed the enzymology and molecular biology of the biosynthesis of fructans, which are commercially important as indigestible sweeteners. Smeeken's group has cloned seven different fructosyltransferase genes from onion; these genes appear to have evolved from vacuolar invertases. Bacterial fructosyltransferase (levan sucrase) was engineered into ten different plant species, including sugar beet. Vacuolar targeting of the enzyme in beet led, unexpectedly, to high concentrations of fructan in the endomembrane system rather than the vacuole (the storage site for sucrose), suggesting that there must be a high concentration of sucrose in the endomembrane system.

Speakers in this session noted examples where attempts to overexpress genes of carbohydrate metabolism in transgenic plants led to cellular necrosis. In the case of plants overexpressing levan sucrase, the high concentration of free glucose released appeared to signal a typical hypersensitive response, including programmed cell death and elevated levels of salicylic acid and pathogenesis-related proteins.

M. Stitt (University of Heidelberg, Heidelberg, Germany) completed the session by considering more global aspects of metabolic regulation in transgenic plants, particularly with respect to the role of nitrate as a signal to regulate shoot–root allocation. A series of transformed tobacco plants with varying levels of nitrate reductase were used to demonstrate that nitrate in the shoots is perceived as a signal for gene expression,

carbon allocation and root development by mechanisms independent of nitrate metabolism *per se*. More whole-plant studies of this type are needed to assess the impact of transgenic modifications to plant primary metabolism.

Genes and controls for lipid synthesis

In the future, it is likely that the products produced in plants by metabolic engineering will become increasingly important in agriculture. J. Ohlrogge (Michigan State University, East Lansing, MI, USA) described one prominent example of a crop now in commercial production with altered seed composition, namely high laurate rapeseed. By the introduction of an acyl-ACP (acyl carrier protein) thioesterase gene from the California Bay plant (a common species in California), rapeseed has been engineered to change its fatty acid composition from long-chain fatty acids to predominantly 12-carbon structures. Such oils have special physical properties that are ideally suited for a variety of industrial and food uses. The success in engineering high-laurate rapeseed indicates that large changes in oil composition can be engineered, resulting in a higher-value product. However, examination of fatty acid metabolism in the high-laurate rapeseed revealed that a substantial proportion of the lauric acid is probably oxidised before it can be incorporated into storage oils. Thus, these seeds seem to have a futile cycle of fatty acid synthesis and oxidation, although, surprisingly, oil yields are not reduced.

The dream of designing entirely new oil compositions is getting closer, as J. Shanklin (Brookhaven National Laboratory, NY, USA) described in his report on the engineering of acyl-ACP desaturases. Site-directed mutagenesis has been used to produce a new enzyme with altered chain-length specificity, and further developments in this area are expected to lead to fatty acids with double bonds in unique locations that may fill specific industrial needs.

J. Kridl (Calgene, Davis, CA, USA) presented Calgene's progress on producing high-stearate rapeseed oils. Such oils might eliminate the need for hydrogenation of vegetable oils for margarine manufacture or may even substitute for cocoa butter, which currently sells for approximately ten times the price of rapeseed oil. The original transgenic lines, produced by antisense of the acyl-ACP desaturase, were found to be unstable in their fatty acid composition. Kridl has now produced a second generation that combine the antisense desaturase with expression of an acyl-ACP thioesterase that preferentially hydrolyses saturated fatty acids. Tests are under way to evaluate the stability of the high-stearate oil composition in these lines.

A general conclusion from this session was that engineering oil composition has been proved to work and to lead to new commercial products. Although a few new products may arise from manipulation of only a single gene, in many cases, achieving high-level production of high-value speciality oils will require multiple gene manipulations.

Cell-wall polymers

This session covered the advances in fundamental and applied research on two of nature's most abundant macromolecules, cellulose and lignin. M. Brown (University of Texas, Austin, TX, USA) described the advances in cellulose structure and biosynthesis, the latter focusing on macromolecular morphology of the synthesising complex and its relation to microfibril assembly. He also described recent advances in cellulose biochemistry, including the *in vitro* synthesis of cellulose I. Because the biochemistry of cellulose synthesis in vascular plants has lagged far behind that in prokaryotes, transgenic plants have not yet been used to study cellulose, but recent advances in the molecular biology of cellulose will obviously close this gap. I. Saxena (University of Texas) reviewed the multidomain architecture of β-glycosyl transferases. Hydrophobic-cluster analysis has defined the conserved residues in β-glycosyl transferases that are potential catalytic residues. So far, a number of sequences have been identified in higher plants that carry these conserved residues; however, this conservation suggests only that these are β-glycosyl transferases, not whether they are involved in cellulose biosynthesis. Using the *Acetobacter* system, it may be possible to identify the function of a number of other important β-glycosyl transferases (e.g. in the synthesis of xyloglucans, xylans, arabinans, rhamnogalacturonans) in the absence of transgenic-plant studies.

The pathway of lignin biosynthesis is generally believed to be much better understood than that of cellulose, and a number of lignin-biosynthetic genes have now been manipulated in transgenic plants, although with somewhat contradictory results. C. Chapple (Purdue University, West Lafayette, IN, USA) discussed the ferulate-5-hydroxylase (F5H) cytochrome P450 and its central role in phenylpropanoid metabolism in *Arabidopsis*. Manipulation of genes early in the lignin biosynthetic pathway offers promise for the control of lignification, which could lead to more economical ways to process wood in the paper-making industry.

As with other presentations in the symposium, everyone came away with the feeling that isolation and manipulation of a single gene in a complex pathway is not sufficient to significantly alter important agriculturally-related characteristics. The future will most certainly require the identification of most of the genes in the cellulose and lignin pathways in order effectively to manipulate cell walls and their properties.

Protein engineering

C. Arntzen (Boyce Thompson Institute for Plant Research) discussed progress in the production of immunogenic proteins in transgenic plants and evaluation of their ability to act as oral vaccines. The most advanced study utilised the binding subunit of the *E. coli* heat-labile enterotoxin (LT-B), which has known value for oral immunisation against bacterial-induced diarrheal disease. Greater than 1.0% of total protein accumulation has been achieved in edible plant tissues for the LT-B; increased accumulation could be achieved through use of a gene encoding a fusion protein containing a microsomal retention signal. When mice were fed raw potatoes expressing the LT-B, serum and secretory antibodies specific for the LT-B were induced. The potato-immunised animals were protected from challenge with authentic bacterial toxin. A request has been submitted to the US Food and Drug Administration to extend these studies to human clinical trials. He reported successful oral immunisation using transgenic potatoes expressing the capsid protein of Norwalk Virus (a causal agent of human diarrheal disease) and described efforts to make a multi-component diarrheal vaccine in a single plant. Successful immunisations using

plant-derived hepatitis-B surface antigen were also reported, as was the progress towards the use of bananas as an expression system for 'edible vaccines'.

J. Ma (Guy's Hospital, London, UK) discussed the expression of IgG and secretory-IgA antibodies in transgenic plants. He described the assembly pathways of antibodies in plants, which appears to match those in mammalian cells. The role of protein chaperones was discussed and, in particular, that of the plant BiP homolog, an endoplasmic-reticulum-resident member of the HSP70 family. These antibody-assembly pathways and mechanisms in plants are supported by the finding of well developed ER and Golgi in fully differentiated leaf cells from transgenic plants.

J. Johnson (Scripps Research Institute, La Jolla, CA, USA) presented work performed in collaboration with colleagues at the John Innes Institute (Norwich, UK) and Axis Genetics Corp. (Cambridge, UK) on structure-based design of peptide presentation on plant-virus surfaces. This included crystallographic studies of a chimera of cowpea mosaic virus (CPMV), in which nucleotides encoding 14 residues of a neutralising immunogenic epitope of human rhinovirus 14 were inserted into an infectious CPMV clone, resulting in the expression of the epitope on an exposed loop of a CPMV capsid subunit. Crystallographic data were also presented on a CPMV chimera based on residues 731–752 of HIV gp41 that indicated a high degree of flexibility of the inserted loop. Antibodies against this chimera neutralised HIV-induced syncytia formation in cell culture; plant-virus presentation provides a context in which the epitope is highly immunogenic.

G. della-Cioppa (BioSource Technologies, Vacaville, CA, USA) described how plant viruses can be developed as episomal vectors for high-level expression of foreign genes in field-grown plants. BioSource has developed viral vectors based on hybrid versions of tobacco mosaic virus and other plus-sense RNA viruses. Hybrid RNA transcripts can be made *in vitro* and then passed through a packaging host in a growth chamber or greenhouse in order to obtain hybrid virion particles. These hybrid viruses can then be taken to the field for large-scale systemic transfection of a desired cultivar. Heterologous RNA transcripts and foreign proteins can accumulate to very high levels (many kilograms per acre) in one to two weeks after transfection with the vector. These transfection systems allow the overproduction of proteins and secondary plant products in leaf tissue in amounts that would be lethal if carried through seed by traditional plant-transformation techniques. Several examples of overproduction of carotenoid-biosynthetic enzymes have proved the basic viral technology for small-molecule pathway engineering in the leaves of mature plants. In 1996, BioSource released 16 recombinant viral vectors into the field and processed approximately 100 tons of leaf biomass.

C. Cramer (Virginia Technical University, Blacksburg, VA, USA) presented research on the post-harvest production of human lysosomal enzymes in the leaves of transgenic tobacco. Two human glycoproteins with potential as replacement-enzyme therapeutics were successfully produced in tobacco and shown to be enzymatically active. Glucocerebrosidase, a treatment for Gaucher disease and currently one of the most expensive drugs in the world, was produced at levels exceeding 10% of soluble protein in transgenic leaf extracts using an inducible expression system; the transgene product is recovered 8–12 hours after induction. A second lysosomal enzyme, iduronidase, which is a soluble glycoprotein, was also secreted in plants and recovered from the induction medium, greatly facilitating purification.

Protein targeting and engineering

The final session of the meeting reviewed recent advances in our ability to target transgene-expressed proteins to specific cellular compartments. E. Krebbers (Du Pont Agricultural Biotechnology, Wilmington, DE, USA) summarised work on engineering high-lysine corn, while K. Keegstra (Michigan State University) described the chloroplast membrane components involved in recognition, transport and processing of peptides containing chloroplast-transit peptides. P. Maliga (Rutgers State University) described the technology of plastid transformation and its advantages. Transforming the plastid genome results in no transmission of the introduced trait via the pollen, high expression levels (owing to the large number of genome copies), no position effects as homologous recombination occurs and no known mechanisms of gene inactivation. Abolishing expression of the nuclear-encoded plastid RNA polymerase determined those genes that are dependent on the nuclear- or plastid-encoded polymerases for their expression, and these studies are leading to the development of tissue-specific plastid promoters as new tools for plant genetic manipulation.

In the final talk of the conference, R. Boston (North Carolina State University, Raleigh, NC, USA) reminded the audience of the importance of molecular chaperones such as GRP94, BiP, calnexin, calreticulin and protein disulfide isomerase for protein processing, using as a model the maize floury 2 mutant, in which incorrect zein processing is accompanied by increased levels of molecular chaperones.

Workshops

In addition to the above sessions, the meeting featured two workshops on emerging technologies. The first, chaired by J. Mol (Free University) and B. Thompson (North Carolina State University) discussed how best to avoid or exploit epigenetic gene suppression and how to stabilise transgene expression by using nuclear-matrix-attachment regions or incorporation of bacterial-artificial-chromosome-derived large-plant-genomic fragments. In the second workshop, chaired by W. Schuch (Zeneca Plant Science, Bracknell, UK), a more holistic approach was taken to the problems of metabolic-pathway engineering, and considerable interest was expressed in the need for databases of plant metabolic parameters that would facilitate computer simulation of pathway fluxes in response to proposed transgene-mediated changes in pathway enzymes.

Acknowledgments

We thank David Baulcombe, Malcolm Brown, Dan Klessig and John Ohlrogge for written summaries of the sessions they chaired, edited versions of which have been incorporated into this report.

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Control of metabolic flux in yeasts and fungi

Meeting report

Most biotechnology companies that exploit microbial fermentations are actively seeking methods of improving the productivity or quality of their fermentations. On the one hand, strain manipulation is becoming easier, as molecular tool boxes grow ever more refined and microbial genome sequence information expands exponentially. On the other hand, the molecular manipulation of metabolic pathways appears to be becoming more complex, as many attempts to increase the flux through specific pathways have met with limited success. Although such problems were predicted many years ago by metabolic control analysis (MCA), many molecular biologists remain blissfully unaware of this area. Therefore, a recent European meeting*, which brought together industrial and academic researchers with expertise ranging from metabolic modelling to molecular genetics, was particularly timely.

Enzymes are democratic – control is shared

Many biotechnologists wish to raise fermentation yields by increasing the flux through a specific biochemical pathway. Old dogma suggested that the best approach is to overexpress a specific rate-limiting enzyme and thereby release a metabolic bottleneck; unfortunately, this popular misconception still litters the textbooks. Hence, in her opening remarks, the Conference Chair, J. M. Gancedo (Instituto de Investigaciones Biomédicas, Madrid, Spain) reminded us that metabolic control is shared between enzymes: enzymes are democratic, not aristocratic. This central principle of MCA was well illustrated by D. Fell (Oxford Brookes University, Oxford, UK), who described how the mathematical modelling that underpins MCA can be used to make firm predictions about how metabolic pathways will respond to manipulation[1]. From a biotechnological point of view, one of the most important predictions is that, in most cases, the overexpression of a single enzyme will exert a minimal effect upon flux. Instead, significant increases in flux are more likely to be achieved by coordinated increases in the levels of groups of enzymes. This principle has been elegantly demonstrated for aromatic amino acid biosynthesis in yeast[2] and penicillin biosynthesis in filamentous fungi.

'All models are wrong, but some are useful'

If MCA is so useful, why has it not been welcomed with open arms by the biotechnology community? The problem, of course, lies in the phobia of mathematical formulae displayed by most biologists. Hence, the answer lies in the generation of user-friendly but powerful software packages that can simulate metabolic pathways accurately but also exploit familiar types of data (e.g. K_m, V_{max}, the Hill coefficient, etc.). The program GEPASI, which is freely available on the WWW (http://gepasi.dbs.aber.ac.uk/softw/gepasi.html), would appear to meet these criteria. P. Mendes (University of Wales, Aberystwyth, UK) described how this program can be used to predict how specific manipulations will change fluxes and also to reveal stoichiometric biochemical relationships that are not intuitively obvious. He pointed out that, even if only limited experimental data are available, computer modelling provides a fast, inexpensive and useful way of identifying which experimental unknowns are the most significant and should be measured first.

Other speakers described the application of specialized mathematical models. As part of a project to define metabolic behaviour in the heterogeneous environment of a large-scale fermenter, M. Reuss (University of Stuttgart, Stuttgart, Germany) modelled the short-term responses of yeast cells to changes in glucose levels and suggested that this technique allows the diagnosis of enzyme kinetics *in vivo*, thereby overcoming the current dependence upon values measured *in vitro*. J. Nielsen's (Technical University of Denmark, Lyngby, Denmark) modelling of penicillin biosynthesis revealed that increased aeration would enhance yields, a prediction that he confirmed experimentally. Similarly, the less-well-known technique of cybernetic modelling is being used on the nitrogen metabolism of yeast in order to facilitate process control of growth for optimal production of yeast flavouring extracts (M. Giuseppin, Unilever, Vlaardingen, The Netherlands). This type of model simulates detailed aspects of system behaviour by inferring short-term kinetic responses, on the basis that they tend to achieve the goals of the yeast cell. Interestingly, the predictive ability of this model was improved significantly by incorporating long-term feedback mechanisms brought about by changes in gene expression. The general conclusion was therefore that accurate metabolic modelling will frequently yield significant process improvements.

'Measurement, discovery, thought, then action'

H. Holms (Bioflux Ltd, Glasgow, UK) presented a useful pragmatic alternative to kinetic modelling. The principle that underpins his method of flux analysis is that, if the metabolic pathways of interest have been identified, the flux though these pathways can be determined by measuring all metabolic inputs, outputs and the levels of key metabolites at critical branch points with other pathways[3]. He described how this straightforward approach has been applied successfully to improve the productivity of a wide range of industrial fermentations, including heterologous protein production. A number of other groups presented further evidence for the validity of this approach.

A central metabolic pathway resists manipulation

Control of glucose transport is complex in yeast. E. Boles (Heinrich-Heine University, Dusseldorf, Germany) discussed 20 members of a hexose-transporter gene family in yeast that have been revealed by genome sequencing. Some control over glucose metabolism resides at the level of transport, and therefore a complete understanding of glucose metabolism in yeast depends upon a detailed dissection of this large gene family. The genes *HXT1–7* are the major players in glucose transport, as

*The European Research Conference on *Approaches for Understanding the Control of Flux in Yeasts and Fungi* was chaired by J. M. Gancedo and organized by the European Science Foundation and held in Giens, France, 14–18 June 1997.

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PII: S0167-7799(97)01116-5

is shown by the fact that the deletion of all of these genes blocks transport. The kinetics of hexose transport by individual Hxt proteins is now being analysed in the Δ*hxt1–7* yeast mutant, revealing a complex interplay between low- and high-affinity transporters. In addition, M. Johnston (Washington University, St Louis, MO, USA) described how *HXT* genes are differentially regulated in response to high- and low-glucose signals. Two members of the family detect these signals, the long C-terminal tails on the Snf3p and Rgt2p proteins being critical for this signalling function. The roles of most other members of the hexose-transporter gene family remain elusive.

In yeast, glucose transport and glycolysis are linked by mechanisms that are not intuitively obvious (J. M. Gancedo). Excess glucose phosphorylation depletes ATP pools, inhibiting growth, and therefore a feedback loop operates to regulate phosphorylation. Similarly, key steps in glycolysis are exquisitely regulated by allosteric loops, so that changes in enzyme levels are accompanied by compensatory changes in enzyme activity. This is why manipulation of phosphofructokinase and pyruvate-kinase levels exerts minimal effects upon flux (A. Brown, Aberdeen University, Aberdeen, UK), another observation that was predicted by MCA[1]. An alternative approach that has met with some success is the manipulation of groups of enzymes by altering the levels of key transcriptional regulators such as Mig1p (L. Olsson, Technical University of Denmark, Lyngby, Denmark) and Hap4p (J. Blom, Biocenter Amsterdam, Amsterdam, The Netherlands). Hap4p overexpression appears to shift the carbon flux towards respiratory metabolism and increases biomass production.

The possibility of physical interactions between glycolytic enzymes complicates the situation. Glycolytic enzymes are very abundant and could form some kind of structure within the yeast cytoplasm; some argue that such interactions might facilitate substrate channelling, although this is an area of some controversy. F. Zimmermann (Technische Hochschule Darmstadt, Darmstadt, Germany) proposed that, given the high concentrations of these enzymes, such interactions might be metabolically significant without being strong enough to detect in two-hybrid screens. He has therefore embarked upon the detailed kinetic analysis of site-directed triose-phosphate-isomerase mutants that have been suggested by molecular modelling. This interesting project is in its early stages, and no such interactions have yet been identified.

Metabolic compartmentation and strain differences present further complications

The product of the glycolytic pathway, pyruvate, can be assimilated into oxaloacetate via pyruvate carboxylase or dissimilated via pyruvate dehydrogenase or pyruvate decarboxylase. The direction of carbon flux at this pyruvate branch point is central to yeast metabolism and its biotechnological exploitation (P. Niederberger, NESTEC SA, Lausanne, Switzerland). Ethanol production must be minimized for efficient production of bakers' yeast, but must be maximized in alcoholic fermentations. Attempts to manipulate carbon flux at this branchpoint are complicated by the compartmentation of glycolysis and the TCA cycle between the cytoplasm and mitochondrion, and by the effects on amino acid biosynthesis of altering the flux of intermediates to the TCA cycle (J. Pronk, Delft University of Technology, Delft, The Netherlands). D. Appling (University of Texas, Austin, TX, USA) described compartmental specialization in C1 metabolism, in which parallel pathways of serine metabolism in the cytoplasm and mitochondrion are used for C1 dissimilation and assimilation, respectively.

An important point was made by P. Niederberger and J. Pronk, who stressed that significant strain differences make it very difficult to extrapolate from academic to industrial yeast strains. After careful analysis, the well-defined CEN.PK family of *Saccharomyces cerevisiae* strains was found to be the best academic model for industrial yeast strains.

Metabolic pathway engineering is easier at the periphery

Several elegant talks illustrated significant successes in the metabolic suburbs. M. Penttila (VTT Biotechnology and Food Research, VTT, Finland) described the introduction of xylose-catabolic enzymes into yeast for commercial ethanol production and for the biosynthesis of the sweetener, xylitol. Xylitol production proved efficient, largely because this involved the introduction of a single enzyme reaction into yeast, but efficient xylose fermentation was more difficult to achieve because xylose catabolism must be tightly integrated with the yeast metabolism. The key problem seems to be the high demand for NADPH and NAD^+ cofactors that disturbs the redox balance; the restoration of the redox balance is likely to improve productivity and yield a commercial process for bioconversion of lignocellulosic hydrolysates.

Malolactic fermentation by lactic-acid bacteria traditionally deacidifies and stabilizes wines, but these fermentations are unreliable. The successful introduction of this malolactic fermentation into yeast depended upon the coexpression of a malate transporter (S. Dequin, INRA, Montpellier, France), illustrating that, after the primary metabolic engineering is performed, adjustments must often be made to achieve the desired efficiencies.

One of the most spectacular successes in metabolic-pathway engineering has been the optimization of penicillin biosynthesis in filamentous fungi. M. Penalva (Centro de Investigaciones Biológicas, Madrid, Spain) presented data on the optimization of this secondary metabolic pathway that illustrated some central principles of MCA. Control is shared – increasing the expression of one enzyme on the pathway immediately reduced its degree of control over the flux and shifted control to other steps in the pathway. Related pathways influence the flux through the pathway of interest – both inactivating an enzyme at a branchpoint another pathway and removing feedback inhibition from an early step in the main pathway diverted flux towards penicillin biosynthesis. However, as described above, J. Nielsen's elegant modelling of this pathway has revealed yet more ways in which penicillin yields can be increased in an industrial strain; MCA can direct the molecular biologist to perform the most appropriate manipulations. Therefore, the rigorous application of metabolic theory and experimentation promises improvements even to efficient biotechnological processes.

What does the future hold?

Potentially, the future of this field is very bright. Academic studies

of metabolic fluxes will increase our understanding of fungal growth and physiology, and, through modelling, these studies will be better focused on the most relevant areas of the metabolic network. Also, the functions of many yeast genes discovered by genome sequencing are more likely to be revealed by developing quantitative analyses of small perturbations in metabolism rather than by searching (often unsuccessfully) for large phenotypic effects in deletion mutants (B. Teusink, UMIST, Manchester, UK). From the biotechnological point of view, metabolic studies will yield clear and testable predictions for the improvement of microbial fermentations. At this meeting, the positive results of this type of close exchange between MCA and molecular biology were clear.

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The use of capillary electrophoresis for point-mutation screening

Keith R. Mitchelson, Jing Cheng and Larry J. Kricka

Advances in capillary electrophoresis technology over the past three years have been rapid. Capillary electrophoresis offers high-throughput, high-resolution, automatic operation and on-line detection with automatic data acquisition, and this has stimulated its application to the analysis of DNA mutations. Many different PCR-based DNA-mutation assays have been developed for unknown and known mutations. This article compares conventional PCR-based mutation-detection assays with the methods developed for use with capillary electrophoresis. Future trends for mutation detection using capillary electrophoresis are also assessed, with a special emphasis on totally integrated, microchip capillary-electrophoresis-based mutation-detection systems.

DNA-mutation detection is important in many fields, including molecular biology, ecology, phylogenetics and medicine, and has become central to the characterization of disease-causing genes and for the diagnosis of human genetic diseases. Several excellent reviews have been published that provide detailed descriptions of the methods developed for the detection of point mutations[1–4]. Most methods (see Table 1 for a guide) can be classified into two general categories: (1) methods to detect known mutations, and (2) methods to detect unknown mutations. Methods for detecting known mutations[2] include techniques such as allele-specific oligonucleotide hybridization, allele-specific amplification (ASA), amplification-refractory-mutation assay (ARMS), ligase chain reaction (LCR), polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) and short tandem repeat (STR) length polymorphism. The methods for detecting unknown mutations[1,2] include single-strand-conformation polymorphism (SSCP)[2,5], heteroduplex-polymorphism assay (HPA), denaturing gradient gel electrophoresis (DGGE)[6], chemical[3] or enzymatic[7,8] cleavage of mismatches (CMC, EMC) and dideoxy fingerprinting (ddF)[9]. Conventionally, mutation assays have employed submerged agarose gels and polyacrylamide-gel electrophoresis (PAGE) for separation of the mutant DNA molecule from the wild-type molecules. Although agarose gels and PAGE are simple and require low-cost equipment, both separation techniques are expensive in terms of time and labour, and neither are particularly amenable to automation. High-resolution, sequencing-quality PAGE equipment has been increasingly used for mutation screening[2,3,5,10], and yet, despite automated data collection, sequencing PAGE remains labour intensive and DNA fractionation requires several hours.

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Capillary electrophoresis

Capillary electrophoresis (CE) was first described in a landmark paper by Jorgenson and Lukacs[11] in 1981. CE instrumentation (Fig. 1) consists of two electrolyte chambers linked by a 50–100 μm internal-diameter capillary. The microcapillary tube facilitates rapid heat dissipation and, despite the application of large electric fields, temperature gradients and distortions, which can affect the resolution of bands in conventional gels, are virtually absent in CE. The technique has been applied widely to the analytical separation of a variety of biopolymers, including proteins, polysaccharides and DNA. An on-line detector positioned close to the outlet of the capillary tube automatically acquires data on fractionated molecules. DNA may be detected during CE analysis using its natural UV absorption. Although UV absorption is sufficient for many molecular-biological applications, its low sensitivity may limit the detection of low-abundance

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PII: S0167-7799(97)01117-7

Table 1. Abbreviations and mutation-detection methods

Summary guide to abbreviations	References to conventional mutation-detection methods	References to analysis using capillary electrophoresis
ALA, allele-specific amplification	1,2	36–39
ARMS, amplification-refractory mutation system	2	29
CAGE, capillary affinity gel electrophoresis	67	67,68
Capillary-array electrophoresis		13,14,37,42,64,65
CDGE, constant-denaturant capillary gel electrophoresis		53,54
CGE, nondenaturing capillary gel electrophoresis		12,17,29,39
Chip capillary electrophoresis		23,24,70,71
CMC, chemical-mismatch cleavage	3,59	
Collection of capillary electrophoresis sample fractions		15,16
CZE, capillary-zone electrophoresis		45,46
ddF, dideoxy fingerprinting	9,49,58	49
DDRT-PCR, differential-display reverse-transcriptase polymerase chain reaction	61,62	
DGGE, denaturing gradient gel electrophoresis	3,6	
DNA sequencing by capillary electrophoresis		14,22,23,69
EMC, enzyme-mismatch cleavage	7,8	
ESCE, entangled-solution capillary electrophoresis		18,19,28,38,43,51,65
HPA, heteroduplex-DNA-polymorphism assay	1,2	43,45,46
LCR, ligase chain reaction	1	33–35,75
LIF, laser-induced fluorescence		11,42,51
Microanalytical device		74–76
PCR–CE, automated polymerase chain reaction and capillary-electrophoresis assay		27,75,76
PCR–RFLP, polymerase chain reaction–restriction fragment length polymorphism	1	30–32
PFCE, pulsed-field capillary electrophoresis		25
QRT-PCR, quantitative reverse-transcriptase polymerase chain reaction	12,56	47
RAPD, randomly amplified polymorphic DNA		66
SSCP, single-stranded-DNA-conformation polymorphism	1,2,5,10,52	26,39–41,50,51
STR, short tandem (microsatellite) repeat	1	40,41,46
Thermally-cycled reactions on a chip		35,72,73,76
TPCE, temperature-programmed capillary electrophoresis		55

species or samples at low DNA concentrations. Laser-induced fluorescence (LIF)[12], in which the fluorescence of additives is induced and detected, provides extremely high sensitivity (approximately 100 times that of UV absorption), greatly extending the lower limit of concentration at which DNA may be detected.

Typically, DNA fragments of up to several kilobases in size can be size-fractionated in less than 20 min by CE. Pulsed-field capillary electrophoresis (PFCE) techniques extend the size range of DNA molecules that can be analysed. However, for routine laboratory analysis of 2–20 kb DNA fragments, conventional agarose electrophoresis is a cheaper and more flexible technique than single-capillary-based CE. The development of capillary-array-based equipment[13,14] (Fig. 2) reduces the cost of CE, and the combination of PFCE and capillary arrays would stimulate the application of CE for the rapid analysis of larger DNA fragments. Recent developments[15,16] also allow the collection of nl–μl sample volumes continuously during electrophoresis, thus extending the utility of CE to analyses that require additional post-fractionation assays, such as DNA hybridization or PCR amplification.

Different electrophoresis media have been developed for CE that provide a range of separation properties. Capillary gel electrophoresis (CGE) adopted the conventional slab-gel electrophoretic methods for CE technology, using a fused-silica capillary column filled with a gel of either polyacrylamide or agarose. CGE has high resolution – short DNA fragments can be fractionated to single-nucleotide resolution sufficient for DNA-sequence analysis[14]. The main disadvantage of using CGE in mutation screening is that the capillary has a limited number of operational runs, as the matrix is eventually prone to destruction by the high current densities. However, the operational life of CGE capillaries (10–15 hr) is still superior to conventional electrophoresis gels, which are newly cast for each analysis, and methods to improve in-column polymerization have been developed[17].

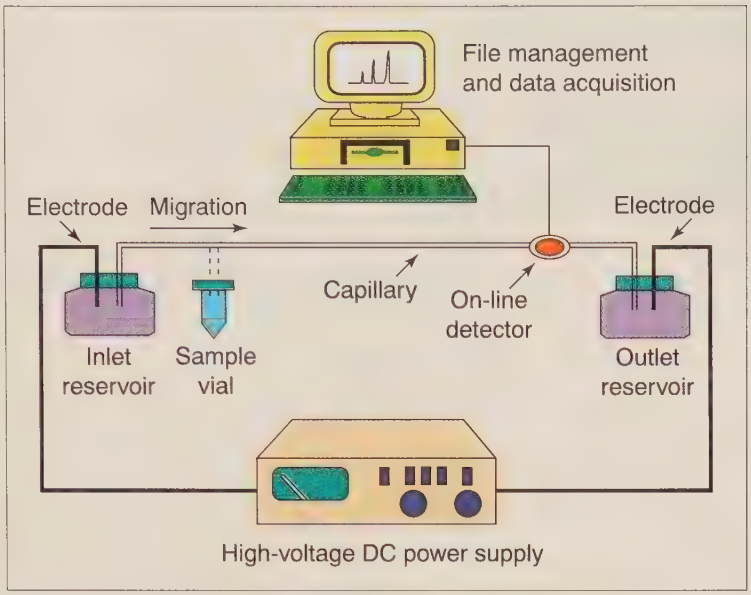

Figure 1
A schematic diagram of a capillary-electrophoresis system. Capillary-electrophoresis instrumentation consists of two electrolyte chambers with a linking 50–100 μm internal-diameter capillary in which a very-high-strength electric field (200 V cm^{-1} to 1000 V cm^{-1}) is applied. Data on fractionated molecules is automatically acquired by an on-line detector positioned close to the outlet of the capillary.

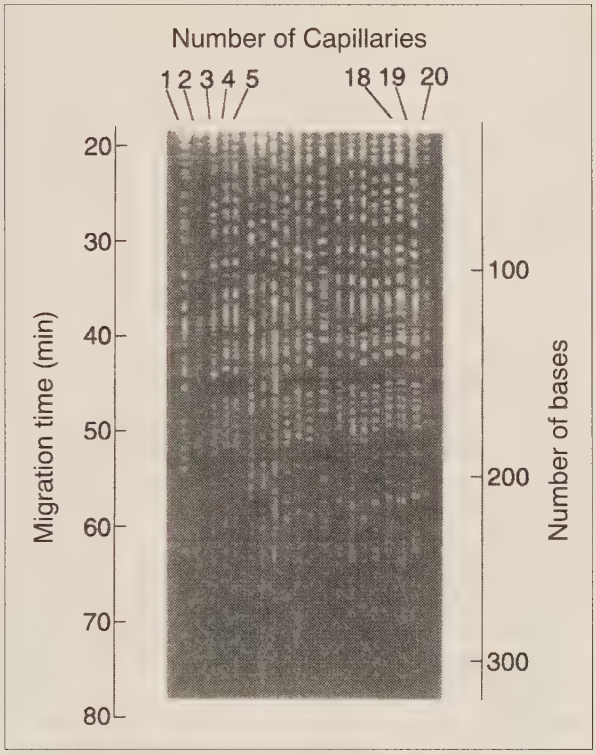

Figure 2
One-colour fluorescence ladder of M13 mp18 'A' DNA sequence products displayed on a 20-channel CGE capillary array. Reprinted from Ref. 14 with permission.

Entangled-solution capillary electrophoresis (ESCE) is a form of CGE that uses an electrophoretic matrix composed of dynamic sieving media[18]. In ESCE, polymers including linear polyacrylamide, polyacrylaminoethoxyethanol, polydextrans, poly(ethylene oxide) or cellulose derivatives such as methylcellulose or hydroxypropylmethylcellulose are used as additives in the electrophoresis buffer[18–22]. These molecules can form a physically or chemically entangled network sufficient to fractionate DNA. The entangled polymers form 'dynamic pores' whose size is dependent on the type and concentration of the polymer, and the migration-buffer composition[18,22]. The flexible entangled polymers do not prevent the movement even of large DNA molecules, but cause molecules to separate according to their size or conformation. ESCE has been used for analyses ranging from single-nucleotide-resolution DNA sequencing[18,22–24] to megabase DNA fractionation using ultradilute buffers[25]. The main advantage of ESCE is that the electrophoresis medium can be automatically replaced after each run by pressurized injection into the capillary, and thus a high electrophoretic reproducibility can be maintained for each run. During ESCE, the DNA sample is injected into the capillary either electrokinetically or by pressure, whereas only the former injection method is possible with CGE. Coated capillaries provide electrophoretic reproducibility for 100 hr or more before it is necessary to replace the capillary, and new dynamic sieving media that may be used with uncoated capillaries offer the potential for increasing the capillary lifetime still further[22].

Mutation detection using capillary electrophoresis

The development of CE for PCR-based DNA-mutation-detection assays has progressed rapidly for both known- and unknown-mutation-detection methodologies (Table 1). Introduction of DNA samples into the capillary in high-ionic-strength solutions can result in anomalous migration[26], and so purified DNA dissolved in water or in a low-ionic-strength buffer is frequently used[27]. However, conveniently, samples amplified in PCR-based reactions can be loaded onto CE columns directly, with little reduction in fragment resolution or increase in separation times[28,29].

Methods to detect known mutations

PCR–restriction fragment length polymorphism

PCR–RFLP[1] is a widely applied technique in known-mutation analysis for DNA fragments of up to several kilobases. The technique detects the availability of a restriction-endonuclease cleavage site at the mutation locus in DNA fragments amplified from a target template by PCR. PCR–RFLP can be analysed by either CGE or ESCE using LIF or UV detection. ESCE has been used in PCR–RFLP for determining the identity of bacterial species[30] and for the analysis of the DNA fragments of the *K-ras* locus, identifying mutant codons that are often found in a large number of human carcinomas and several subtypes of leukaemia[31]. Fragment resolution using CE is generally superior to conventional agarose-gel electrophoresis. For example, the 271 and 281 base-pair

fragments from an *Hae*III digest of ΦX174 (a common DNA size marker) can be resolved into two discrete peaks by CE[19,21] but cannot be resolved by conventional agarose-gel electrophoresis. Furthermore, high field strength and short capillary lengths allowed ESCE analysis of two DNA fragments of 375 and 400 bp in 60 sec[32], an achievement far superior to conventional agarose-gel electrophoresis and approaching the analysis speeds achieved by CE on a microchip (see later).

Ligase chain reaction

The LCR is a mutation-detection reaction followed by signal amplification that is gaining importance in the routine analysis of known mutations that cause human disease[1]. Two pairs of complementary oligonucleotide probes are used in the reaction; if the probe sequences match the target sequences perfectly, then the probes will hybridize in juxtaposition on each of the target segments and become ligated, but if a point mutation occurs in the targeted segments then no ligation products will be produced. ESCE has been used for the rapid analysis of multiplexed LCR products in the diagnosis of cystic fibrosis[33] and Leber's hereditary optic neuropathy[34]. The time required for CE analysis is almost ten times less than that for a polyacrylamide-slab-gel analysis with comparable resolution. A sensitive fluorescence system is ideally suited to the examination of LCR amplification in a microfabricated silicon-glass chip (Fig. 3). Miniaturization of the LCR and its subsequent analysis using ESCE will increase the sensitivity and speed of analysis[35]; microscale LCR analysis may also be applicable to the detection of UV-photodamaged DNA products.

Allele-specific amplification

ASA is a mutation-detection assay in which two oligonucleotide probes are used in a PCR reaction; if the probe sequences complement the target allele sequences perfectly, then the probes will hybridize on the target segments and become PCR-amplified, but if a point mutation occurs in the targeted segments then no amplification products will be produced[1]. Techniques that provide a quantitative estimation of allele dosage are necessary for the ASA assay. CGE has been used for rapid analysis of ASA genotyping reactions in both single-capillary[36] and capillary-array formats[37]. Multiplex PCR amplification of several potential mutation loci is commonly used to determine the mutant-gene carrier status of individuals[1]. Quantitative LIF detection of 16 out of 18 of the multiplex-PCR-amplification products of the Duchenne–Becker muscular dystrophy gene was achieved in less than 10 min using ESCE with a sieving polymer solution of hydroxypropylmethylcellulose and glycerol[38] – compared with 70 min reported with the use of a low-viscosity polyacrylamide solution[39]. The combination of quantitative multiplex PCR amplification with capillary-array CE analysis would provide higher throughput capacity and speed than any of the currently used diagnostic systems.

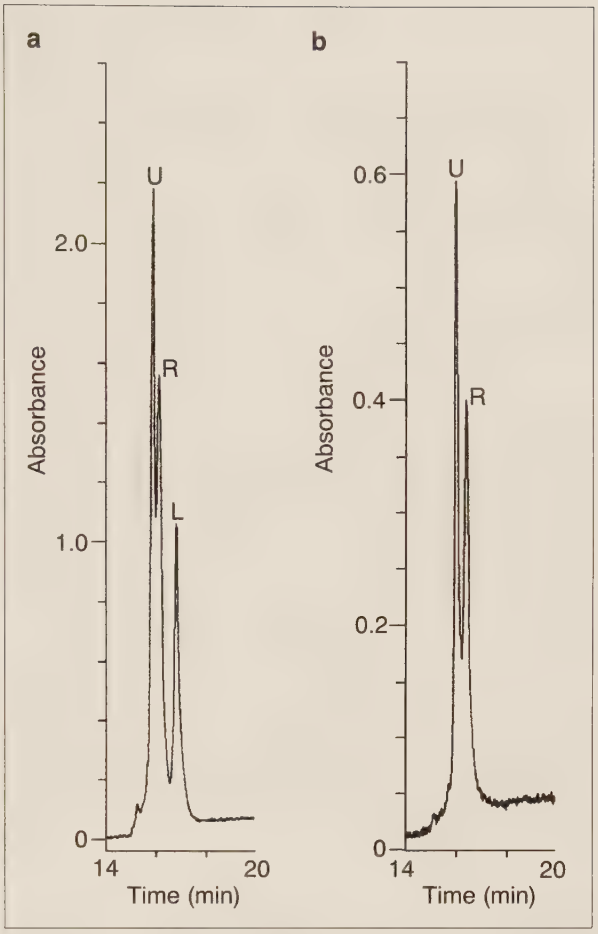

Figure 3

Entangled-solution capillary electrophoresis electropherogram of ligase-chain-reaction products amplified in a silicon-glass microchamber and detected using laser-induced fluorescence[35]. (**a**) Positive reaction. (**b**) Control reaction minus template. The peaks represent unpaired, single-stranded probes (U) (25 nucleotides), reannealed duplex probes (R) (25 bp) and ligase-chain-reaction-amplified double-stranded products (L) (50 bp). Reprinted from Ref. 35 with permission.

ARMS-PCR and STR-PCR

The ARMS-PCR and STR-PCR techniques are increasingly important diagnostic tests for known mutations[1] and are used in the diagnosis of certain inherited diseases and cancers. Both assays involve PCR amplification of specific genomic loci using primers that discriminate either between single-base mutations (ARMS-PCR) or between different numbers of tandem repetitions at a simple sequence locus (STR-PCR). Both CGE[29] and ESCE can detect single-base substitution in genes amplified by ARMS-PCR. The choice of appropriate PCR-primer pairs allows simultaneous multiplexing of reactions for several mutation loci; well-resolved DNA fragments can be obtained by appropriate primer choices.

Some problems in the resolution of STR-PCR alleles have been encountered with samples from heterozygotes. These include poor amplification of the larger allele and PCR-amplification stutter (i.e. amplification of nonspecific fragments), both of which result in diffuse or poorly amplified bands that require

autoradiographic detection following resolution on conventional electrophoresis gels. Reports of ESCE with LIF detection achieving almost base-line resolution of microsatellite 4-bp-repeat alleles in PCR fragments of 122–142 bp within 10 min[40] provide an indication of the tremendous utility of this CE method. The resolution of DNA fragments achievable by ESCE is better than conventional agarose-gel electrophoresis (e.g. 4 bp between 122 bp and 126 bp)[40], while the automatic matrix exchange and higher reproducibility add convenience to its use. A simple CAG-repeat expansion associated with Kennedy's disease and a GATT expansion associated with the cystic-fibrosis gene have been readily detected using CGE, but, generally, CE fractionation of larger STR-expansion alleles has not been reported[41]. The high sensitivity of LIF and fragment resolution of CE may ultimately provide a useful alternative to the gel electrophoresis and autoradiography conventionally used for microsatellite STR detection, particularly if labelled allelic standards are coelectrophoresed with unknown STR alleles[41,42]. The advantage of CE over conventional methods for the forensic analysis of STR alleles and restricted DNA fragments has been suggested, because here the speed of analysis is important[40], and the high throughput of CE would also be advantageous during genetic mapping programs with multiple STR loci[41].

Methods to detect unknown mutations

Heteroduplex polymorphism assay

The HPA is widely used for detecting unknown mutations[1,2]. This technique involves the denaturation of allelic (PCR-amplified) DNA fragments and the subsequent reannealing of the single-stranded DNA molecules to give a mixture of both wild-type and mutant double-stranded DNA (dsDNA) fragments. The resulting products are a mixture of the reassociated homoduplex dsDNAs and novel heteroduplex dsDNAs. The presence of the mismatches within the heteroduplexes results in conformational changes, and so the electrophoretic mobility of the heteroduplexes is retarded relative to the homoduplex in an electrophoretic gel. HPA depends on local conformational changes to duplex DNA, and so the sensitivity decreases with the increase in both DNA-fragment length and the GC content of the region surrounding the mismatch. However, in contrast to SSCP[2] (see later), all heteroduplex mismatches can potentially affect the local DNA structure.

HPA has been performed using ESCE with glycerol and ethidium bromide as additives[43]; the addition of glycerol improves the resolution both by forming pores of various sizes with the cellulose derivatives in the presence of boronic acid and by increasing the viscosity of the medium, and the addition of low concentrations of ethidium bromide exaggerates the structural differences between heteroduplex and homoduplex molecules by intercalating selectively into G–C pairings of DNA molecules. This method differentiated 125 bp heteroduplex DNA fragments from homoduplex fragments that differed by only a single base-pair mismatch. It can be used for rapidly screening DNA mutations, regardless of the sequence of the DNA molecules. A model template DNA[44] with deletions or substitutions can be added as a reference for hybridization with the sample DNAs. This introduces further perturbations into the duplex DNA structure and thus can magnify the conformational effect of the original mutation, facilitating mutation detection by CE. More recently, linear acrylamide has been used as the entangled agent in ESCE to fractionate 3-bp variant alleles of the ΔF508 cystic-fibrosis locus[45] and a GATT microsatellite associated with cystic-fibrosis alleles[46]. Heteroduplex DNAs are produced as a result of the cyclic melting and reannealing of DNAs during a PCR reaction with heterozygous templates. Typically, clear fractionation of heteroduplex molecules was achieved in less than 60 min in addition to separation of the two allelic homoduplexes present in the mixture of PCR products. In conventional HPA analyses, polyacrylamide-gel electrophoresis is used to achieve a similar high resolution of closely similar molecular species, but these assays take approximately four hours to complete, and electrical heating of PAGE gels limits the resolution[2]. The excellent reproducibility and the ease and rapidity of mutation analysis using CE offer a superior format to conventional gel-based techniques.

Single-strand-conformation polymorphism

SSCP is also widely used for unknown-mutation detection[2,5]. It involves dissociation of the double-stranded DNA fragment, after which each of the two single-stranded fragments assumes a folded conformation determined by the specific nucleotide sequence. The sensitivity of SSCP analysis depends on whether the mutation affects the folding of the DNA, and hence the electrophoretic mobility of the single-stranded DNA (ssDNA) molecules. The detection rates for SSCP have been reported to vary widely (from 35% to nearly 100%) depending on whether the mutation existed within or outside the folded single-stranded loop[2]. The position of the mutated nucleotide within the secondary structure of the DNA can affect the detection of sequence changes. Nucleotide variants occurring within single-stranded loops may have no detectable effects on DNA conformation, and consequently this may limit this detection system.

The upper limit on the size of DNA molecules beyond which a single-strand conformational polymorphism cannot be detected has not yet been determined for CE. In a similar way to conventional PAGE analysis of SSCP, the addition of glycerol to ESCE buffers can improve the resolution of SSCP analysis because of additional stabilization of the ssDNA secondary structure[28,47,48]. ESCE has been successfully applied to the detection of single point mutations in *Mycobacterium tuberculosis*-specific amplified DNA fragments with sizes of 219 bp and 363 bp[49]. A study of point mutations in 372 bp PCR products from the

p53 tumour-suppressor gene from patients with multiple myeloma was also successful using nondenaturing CGE[50]. Multiple-fluorescence-based SSCP analysis[47] (multiple colours in a single run) has been reported using ESCE with glycerol to detect mutations in p53 genes[48]. Mutated DNA molecules with similar mobility but different fluorescent tags can be distinguished by their different signals. Despite the efficiency of the multitrack polyacrylamide-gel sequencing technique, the analysis requires an electrophoresis time of 9 hr with tight regulation of the temperature throughout[10], whereas the CE analysis was complete within 11 to 25 min[47–49,51]. Capillary-array CE equipment (Fig. 2), providing simultaneous parallel analysis of up to several hundred different samples[13,14] with run times of less than one hour, is more efficient than analysis using sequencing gels, especially when a multicolour fluorescence detection system is used[14,42].

Metastable conformational isomers of SSCPs (mSSCPs) can form between isolated single strands, and these can be resolved by ESCE[28]. Analysis of mSSCP may extend the screening for point mutations by CE systems. Current limitations of SSCP analysis of DNA fragments centre on the stability of the single-stranded DNA isomers and the resolution of conformational isomers by electrophoretic methods. Magnetic-bead technology[5] allows ssDNAs with identical structure to be isolated, prevents duplexes reforming and provides a stable single-strand conformational polymorphism resource that can be analysed after storage for at least several days[28]. The stability of ssDNA conformations under these conditions would enable high-throughput screening and aid the handling of multiple samples. The accuracy and sensitivity of the assay is also greatly improved because the sample preparation ensures that ssDNAs, including metastable ones, are not destroyed and that migration differences between peaks are maintained. Metastable conformational isomers can also be resolved by conventional polyacrylamide gels[52], but this takes considerably longer to perform.

Constant-denaturant capillary electrophoresis

CDCE[53] is a modified version of DGGE[6] that has wide application in unknown-mutation detection. The dsDNA to be analysed must contain two contrasting domains, a high-melting-temperature region with a length of 50 or more G–C base pairs and a low-melting-temperature region containing the heteroduplex DNA mismatch. During DGGE, the heteroduplex migrates into a gel containing a concentration gradient of one or more denaturants. The dissociation of the DNA heteroduplex initiates at the point where the mismatch(es) are present, then extends rapidly throughout the low-melting-point region and results in a greatly reduced electrophoretic mobility. Although the detection rate of DGGE is unknown, it could theoretically approach 100%.

CDCE (Fig. 4) has been developed for the differentiation of DNA fragments harbouring a single-base

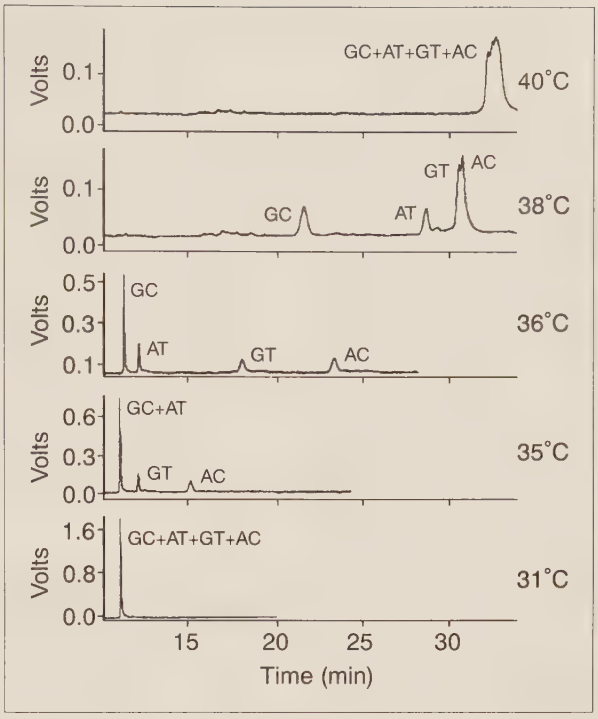

Figure 4

Constant-denaturant capillary-electrophoretic separation of mismatched heteroduplex DNAs as a function of column temperature, as described by Khrapko *et al.*[53] Reprinted from Ref. 53 with permission.

mismatch using a fixed concentration of denaturants (urea and formamide) combined with a constant temperature that can be raised for subsequent runs[53], or a variable temperature that can be increased during a run using computer-controlled thermal ramping[54]. These methods are very sensitive for single-base mismatches, but the direct applicability of this method is determined by the sequence information, that is, whether there is a GC-rich region present in the DNA fragment. It is clear that, once a GC clamp (a GC repeat incorporated at the 5′ end of one of the PCR primers) is attached to either PCR-amplified products[6] or restriction fragments[1], the presence of any mismatches can be easily detected by these CDCE techniques. However, the need for repeated runs for each DNA sample to determine the melting temperature presently limits the utility of CDCE. The critical temperature displayed by individual mismatched DNAs during CDCE would also limit the ease of sequential analysis of DNA fragments differing in size or sequence, although the development of thermal-ramping protocols may overcome these limitations[54]. Temperature-programmed CE is one such approach, using time-programmed thermal gradients to melt all DNA species[55].

Quantitative reverse-transcriptase PCR

In disease states, the activity of particular mutant alleles may influence the pathology of the condition. Quantitative reverse-transcriptase PCR (QRT-PCR) is used for estimating the activity and expression of genes by the synthesis of cDNA

from mRNA followed by quantitative amplification of the cDNA by PCR[56]. The cDNA products of characteristic alleles are conventionally detected by gel electrophoresis and quantified either by autoradiography or by scanning densitometry against internally amplified cDNA standards. Although automated DNA sequencers have been applied to QRT-PCR analysis[56], on-line detection, peak-area quantification and automated data analysis by CE[57] can provide direct analysis of RNA-PCR reactions that reflect *in vivo* gene expression. Importantly, the direct quantification of DNA by its UV absorption provides improved reliability and accuracy compared with the indirect data obtained by scanning a stained PAGE gel or autoradiogram. Data obtained with CE analysis is comparable to that obtained by conventional electrophoresis[12], but it is expected that the greatly improved capacity of CE systems over conventional electrophoresis systems will allow accurate, high-throughput comparison of cDNAs.

Dideoxy fingerprinting

ddF is a method for detecting unknown mutations that combines SSCP and Sanger dideoxy sequencing[9], and can theoretically detect all mutations. In ddF, the products of a Sanger sequencing reaction performed with one dideoxy terminator are electrophoresed through a nondenaturing gel. Mutations can be detected as a result of the gain or loss of a dideoxy termination segment (dideoxy component) and/or by an alteration in the mobility of at least one of the termination segments that contain the mutation (SSCP component)[58]. Nondenaturing CGE has been applied to the detection of single point mutations in a *M. tuberculosis*-specific amplified DNA fragment using ddF[49], and analysis of a single sample was accomplished in less than 25 min. A comparative study indicated that 30 samples could be analysed in 15 hr by single-capillary CGE, whereas conventional polyacrylamide-slab-gel electrophoresis required 36 hr.

Recent and potential applications of capillary electrophoresis to mutation detection

CE is an emerging technology and its application to the detection of DNA mutation is a recent and rapidly developing area of research. In the following sections, some of the mutation-detection methods that can make use of CE are discussed. See Table 2 for a summary of the application of CE to mutation detection with comparisons to conventional electrophoretic analysis.

Enzyme- and chemical-mismatch cleavage

Promising new methods for the identification of point mutations involving enzymatic cleavage of DNA mismatches have been independently reported by Sklar[8] and Cotton[7]. Sklar's enzyme-mismatch cleavage (EMC) uses bacteriophage T4 or T7 endonucleases to cleave heteroduplex DNAs at single-nucleotide mismatches. Both of these enzymatic procedures identified the majority of possible mismatch combinations, and preliminary evidence indicates that crude PCR products can be analysed directly by EMC[8]. With both methods, the low-abundance cleavage products were separated by polyacrylamide-gel electrophoresis and detected by autoradiography. Although these cleavage products have yet to be analysed by CE, the high sensitivity provided by LIF may provide an alternative to autoradiographic detection, although nonspecific cleavage products may increase the background in CE electrophoresis. Because the cleavage methods generate two discrete DNA fragments, the introduction of differential fluorescent tagging provides independent information about the approximate position of a mismatch within the DNA fragment[8]. Also, because the cleaved DNAs are smaller than the original sample DNA fragments, the size of the fragments examined can increase to the limit of the electrophoretic resolution of both PAGE and CE. Both CGE and ESCE can fractionate native DNA to about 10–20 bp resolution for fragment sizes up to 0.5 kb and can resolve fragments up to 20 kb with a resolution of several hundred base pairs. The presence of natural DNA polymorphisms between individuals limits the extension of DNA-cleavage analysis to alleles of defined coding regions, where mismatches, which are generally limited to heteroduplexes associated with disease states, could be used for diagnosis.

Cotton[3] and Prockop[59] have independently developed chemical cleavage of DNA at positions of nucleotide variation in heteroduplex DNAs. Both chemical- and enzymatic-cleavage methods can detect all possible point mutations with an efficiency approaching 100%, but, despite the efficiency of chemical cleavage, its use is less attractive than enzyme systems because of the lengthy cleavage reactions and the use of toxic chemicals. Nevertheless, the lower background of chemical cleavage was used effectively in conjunction with differential fluorescence labelling of PCR primers to allow simultaneous high-quality analysis of different cleavage reactions using an automated DNA sequencer[60].

Differential-display reverse-transcriptase PCR

Differential-display reverse-transcriptase PCR (DDRT-PCR)[61,62] is a technique rapidly gaining popularity for the identification and comparison of the relative levels of gene expression under different conditions by analysing mRNA fragments in the tissues that have been subjected to the different conditions. The transcribed cDNA fragments are typically radiolabelled during PCR amplification and revealed on polyacrylamide sequencing gels. Although the analysis of DDRT-PCR on CE systems has not yet been reported, the high-quality sequencing resolution of DNA fragments reported using CGE[13,14] and ESCE with poly(ethylene oxide)[22] suggests that CE could provide comparable quality to sequencing PAGE, but with greater speed.

Table 2. Comparison of capillary electrophoresis with conventional electrophoresis for use in mutation-detection assays

Mutation assay	Capillary electrophoresis	Conventional electrophoresis
Small DNA fragment assays: restriction fragment variants, ligase chain reaction, etc.	High resolution of fragments from 25 bp to 2 kb. Rapid 5–15 min assay. Detect DNA by direct UV absorption or dye fluorescence.	High resolution of fragments from 300 bp to 10 kb. Slow 15–90 min assay. Detect DNA by direct dye fluorescence.
Heteroduplex polymorphism assay and single-stranded conformation polymorphism assay	High resolution of fragments from 25 bp to 400 bp. Rapid 5–25 min assay. Detect DNA by direct UV absorption or dye fluorescence.	High resolution of fragments from 50 bp to 400 bp. Slow 3–9 hr polyacrylamide-gel assay. Detect DNA by direct dye fluorescence.
Microsatellite-repeat or short-tandem-repeat assay	High resolution of fragments from 50 bp to 500 bp. Rapid 5–25 min assay. Detect DNA by dye fluorescence. Weak alleles are poorly detected.	High resolution of fragments from 50 bp to 400 bp. Slow 3–9 hr polyacrylamide-gel assay. Detect DNA by direct dye fluorescence or by autoradiography. Very sensitive assay.
Chromosomes and large DNA fragments	Pulsed-field capillary electrophoresis: High resolution of fragments from 10 kb to 5 Mb. Rapid 10 min to 4 hr assay. Detect DNA by dye fluorescence or UV absorbance. Collection of fractions may allow DNA sequence analysis or hybridization assay.	Pulsed-field gel electrophoresis: High resolution of fragments from 10 kb to 5 Mb. Slow 2 hr to >100 hr assay. DNA detected by direct dye fluorescence. Genes detected in blotted DNA with high sensitivity.
DNA sequencing	High resolution of fragments from 30 bp to 1 kb. Rapid 10–60 min assay. Detect DNA by direct laser-induced fluorescence with high sensitivity. Suited to capillary-array analysis.	High resolution of fragments from 50 bp to 1 kb. Slow 4–10 hr polyacrylamide-gel assay. Detect DNA by direct dye fluorescence or by indirect silver stain or autoradiography.
Complex DNA mixes: Randomly amplified polymorphic DNA	High resolution of fragments from 30 bp to 1.3 kb. Rapid 5–30 min assay. Detect DNA by direct laser-induced fluorescence with high sensitivity or by UV absorption.	High resolution of fragments from 150 bp to 3 kb. Slow 2 hr agarose-gel assay. Detect DNA by dye fluorescence or silver stain. Simultaneous assay of 30–60 samples
DNA-mutation-cleavage assay: chemical (CMC) enzymic (EMC)	High resolution of fragments from 30 bp to 1 kb. Rapid 10–30 min assay. Detect DNA by direct UV absorption or dye fluorescence. Partial DNA cleavage may cause problems. Suited to capillary-array analysis.	High resolution of fragments from 30 bp to 1 kb. Slow 3–5 hr polyacrylamide-gel or agarose gel assay. Detect DNA by dye fluorescence. Simultaneous assay of 20–30 samples.
Capillary affinity gel electrophoresis	Rapid resolution of specific DNA–protein complexes in 15–25 min. Quantitative detection of DNA by direct UV absorption.	Slow 3–5 hr polyacrylamide-gel or agarose-gel assay. DNA–protein complex detected by indirect stain or autoradiography.

Pulsed-field capillary electrophoresis

Conventional pulsed-field gel electrophoresis (PFGE) requires 1–3 days for the resolution of DNA molecules up to 5.6 Mb in length. The recent development of PFCE, capable of separating large DNA molecules such as *Saccharomyces cerevisiae* chromosomes[25] (Fig. 5) in less than 15 min using ultradilute sieving solutions, greatly extends both the range of fragment sizes capable of resolution by CE and the speed of fractionation compared to conventional PFGE. The restriction analysis of genomes, as used for identification and genotyping of pathogenic prokaryotes[63], would be greatly improved by the introduction of more rapid and less sample-intensive techniques. PFCE has conspicuous advantages and, because nanolitre fractions can be collected using specialized CE equipment[15,16], this may lead to a wider adoption of CE in DNA analysis.

Capillary-array electrophoresis

Several capillary-array-electrophoresis systems are under development, in which the simultaneous analysis of arrays of 96 or more capillaries[13,14,37,42,64,65] will markedly increase the sample throughput. Coupling this increased sample-handling capacity with LIF and differential fluorescence tagging[14] will increase the sensitivity of analysis and the number of samples analysed, respectively. The combination of capillary-array electrophoresis with differential tagging may improve the throughput efficiency by several orders of

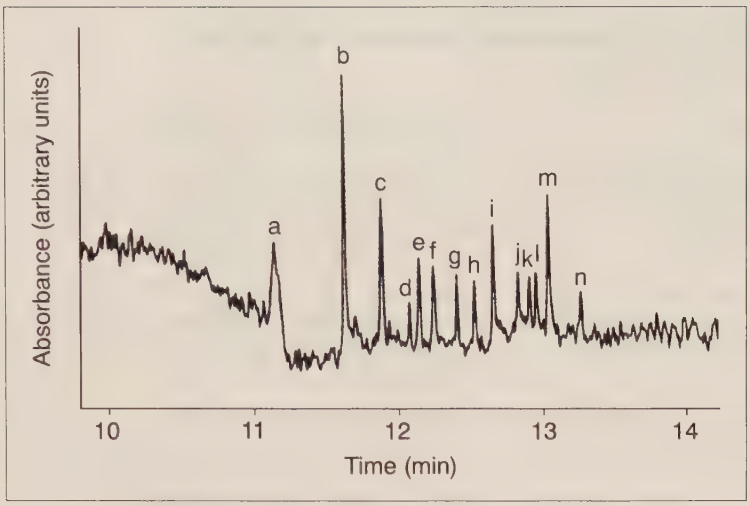

Figure 5
Separation of megabase DNA fragments in an ultradilute entangled-solution capillary electrophoresis buffer using pulsed-field capillary electrophoresis, as reported by Kim and Morris[25]. Peaks are (in Mb) 0.21 (a), 0.28 (b), 0.35 (c), 0.44 (d), 0.55 (e), 0.60 (f), 0.68 (g), 0.75 (h), 0.79 (i), 0.83 (j), 0.94 (k), 0.97 (l), 1.10 and 1.12 (m) and 1.6 (n). Reprinted from Ref. 25 with permission.

magnitude above current single-column CE. New fluorescent dyes[62] can also introduce selective discrimination of tagged DNA products. Capillary-array electrophoresis also has applications in routine screening for common genetic disorders[37,42]. The rapid analysis of human STR alleles was achieved using LIF-induced detection of a two-colour fluorescein-and-rhodamine-labelled primer system on multiple capillary arrays, providing the capacity for simultaneous analysis of multiple samples[42]. Two STR alleles could be separated within 20 min in the same electrophoresis run, giving an accuracy of 0.5 bp or better over 180–200 bp; the laser-induced signals were collected by a scanning confocal microscope. Robotic equipment capable of manipulating microtitre-plate arrays of samples allows rapid switching between different analyses and is already provided on some commercially available systems. The development of these accurate, flexible and rapid analysis techniques will find diagnostic, forensic and genetic applications, especially where there is need for high throughput – for example during high-resolution genetic mapping using randomly amplified polymorphic DNA (RAPD) technology, in which the higher fragment resolution and detection sensitivity of CE provide greater accuracy in genotyping[66].

Capillary affinity gel electrophoresis

Capillary affinity gel electrophoresis (CAGE) is a potentially powerful new approach for DNA mutation detection that uses the specificity of antibodies to select defined DNA sequences or structures combined with rapid analysis by CE for the recognition of specific DNA bases or sequences[67]. Similarly, the protein-mobility-shift assay using CE[68] utilizes specific DNA–protein interactions to identify proteins of interest by the retardation of electrophoretic mobility of the complex. Both of these techniques benefit particularly from the direct quantification of DNA during CE compared with the indirect data obtained from a stained PAGE gel or autoradiogram.

Future trends

DNA sequencing by capillary electrophoresis

The stimulus to DNA sequencing provided by the Human Genome Project and other international genome sequencing projects (yeast, bacteria, plants) has accelerated the development of high-throughput DNA-sequencing strategies. DNA sequencing using conventional planar PAGE gels and ultrathin sequencing gels, despite automatic data collection, is labour intensive and requires electrophoresis runs from 2 to 12 hr for sequence reads of 600–1200 bases. By contrast, DNA sequencing by CE can achieve sequence reads of more than 1000 bases within 80 min using ESCE[69]. High-capacity capillary-array electrophoresis is an attractive alternative to conventional planar gels for DNA sequencing (Fig. 2), and would further increase the sequence throughput.

Capillary electrophoresis on a microchip

Recently, high molecular weight DNA was digested by a restriction endonuclease and then separated electrophoretically in channels on a microfabricated chip consisting of two glass–glass layers[70]. The separation of a series of DNA fragments ranging from 75 to 1632 bp took only 150 sec. A similar analysis of two PCR–RFLP fragments of 440 and 1075 bp took 140 sec (Fig. 6). This was ten times quicker than conventional CE, and the immense reduction in the size of the

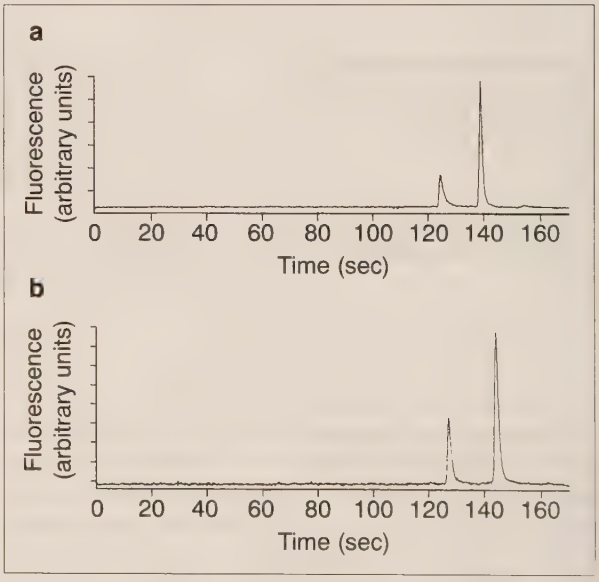

Figure 6
Polymerase chain reaction–restriction fragment length polymorphism products generated in a silicon-glass chip and then detected by capillary-electrophoresis on a glass–glass chip. (**a**) Control, tube-amplified PCR–RFLP products and (**b**) chip-amplified PCR–RFLP products. The two *Hin*fI-restricted PCR fragments are 440 (left) and 1075 (right) base pairs long. The electrophoresis buffer was linear polyacrylamide and the DNA products were detected using laser-induced fluorescence.

apparatus and the considerable saving in both reagent and sample (submicrolitre level) are significant advantages. Ultra-high-speed analysis of DNA fragments ranging from dsDNA-PCR products[24,71] to DNA-sequencing products[23] has also been demonstrated on microfabricated capillary-array-electrophoresis chips. The integration of thermally-cycled amplification reactions[35,72,73] and capillary electrophoresis[23,71] on a disposable silicon chip to create complete microanalytical devices[70,74–76] will bring the analytical possibilities of CE to analytical and research scientists in a pocket-sized format. Such devices have achieved PCR amplification in 15 min and CE in 2 min, to provide complete analysis in under 20 min[75]. Real-time monitoring of PCR amplification reactions with integrated microanalytical devices will allow the direct quantification of gene dosage by PCR–RFLP analysis and quantification of gene expression by QRT-PCR analysis.

CE technologies that will be particularly important for further research and development include improving the automation, throughput, reliability and sensitivity of CE analysis. The recent development of low-viscosity, replaceable sieving matrices[18,22,65] that enhance the run-to-run reproducibility of CE will, combined with the improvement of the speed and efficiency of CE analysis with shorter capillary lengths[32] and capillary arrays[14,65], markedly increase the throughput capacity of DNA analysis by conventional CE. The automation and integration of PCR and CE analysis[27,75] will also contribute greatly to its adoption as the analysis tool of choice for DNA sequencing[14,69], genome mapping[66], disease detection[76] and DNA-mutation analysis[4,67].

Acknowledgments

We wish to thank Ian Clark for encouragement and to acknowledge the support of Beckman Instruments Inc. (UK) and the Scottish Office.

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Bacterial *N*-acyl-homoserine-lactone-dependent signalling and its potential biotechnological applications

Neil D. Robson, Anthony R. J. Cox, Simon J. McGowan, Barrie W. Bycroft and George P. C. Salmond

N-acyl homoserine lactones are bacterial signalling molecules involved in regulating diverse metabolic functions, particularly those relating to virulence, in concert with cell density. Each aspect of the signalling pathway, from production and recognition of the signal to expression of the target genes, offers a potential opportunity for exploitation. Attention is now focusing on the development of novel methods for bacterial enumeration, modulation of bacterial virulence and flexible, coordinated expression of heterologous genes through the use of *N*-acyl-homoserine-lactone-based systems.

A single pathogenic bacterium attempting to invade its host usually stands little chance of mounting a sufficiently strong attack to overcome that host's defences. Such a pathogen would benefit from having a means of coordinating expression of the required virulence factors with other members of its species, and of avoiding mounting an attack until sufficient numbers are present. Pathogenic bacteria from a number of different genera appear to have developed control systems of the so-called LuxI/LuxR type for this purpose (Table 1). The details of the LuxI/R system itself have recently been reviewed[16] and will not be described in detail here. The strategy of linking the production of virulence factors to the overall number of cells has been termed 'quorum sensing'[17]. Bacteria using this system each produce a signal, an *N*-acyl homoserine lactone (AHL), as they grow. In a confined environment, where the otherwise freely diffusible AHL cannot leak away, it will accumulate. The ability to sense

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PII: S0167-7799(97)01102-5

Table 1. Bacterial *N*-acyl homoserine lactones and their associated phenotypes

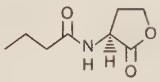
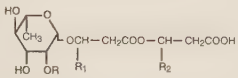
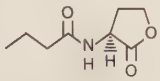
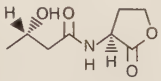
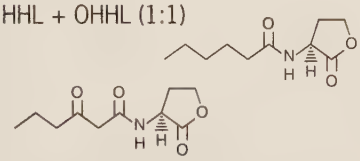
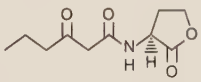
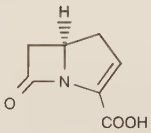
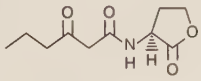
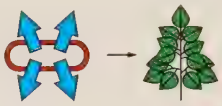
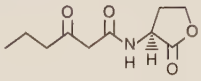
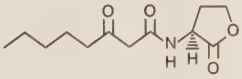
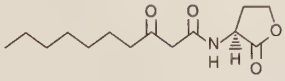
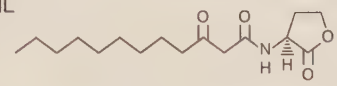
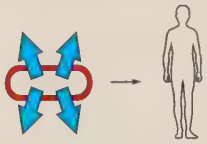
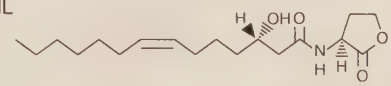
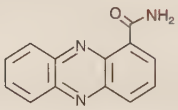
Organism	Major signalling molecule	Phenotype(s)
Pseudomonas aeruginosa	BHL   *N*-butanoyl-L-homoserine lactone	Virulence factors including rhamnolipid, alkaline protease, cyanide, elastase, haemolysin, pyocyanin[1]
Serratia liquefaciens	BHL  *N*-butanoyl-L-homoserine lactone	Swarming: initiation of cell differentiation to produce hyperflagellated, extremely motile 'swarmer cells'[2]
Vibrio harveyi	HBHL  *N*-(3-hydroxybutanoyl)-L-homoserine lactone	Bioluminescence[3]
Yersinia enterocolitica	HHL + OHHL (1:1)  *N*-hexanoyl-L-homoserine lactone + *N*-(3-oxohexanoyl)-L-homoserine lactone	Unknown[4]
Erwinia carotovora	OHHL   *N*-(3-oxohexanoyl)-L-homoserine lactone	(5*R*)-Carbapen-2-em-3-carboxylic-acid antibiotic[5,6]
Erwinia carotovora (*Eca*) and *Erwinia stewartii* (*Est*)	OHHL   *N*-(3-oxohexanoyl)-L-homoserine lactone	Virulence factors: protease, cellulases, pectinases[7] (*Eca*); exopolysaccharide (*Est*)[8]
Photobacterium fischeri	OHHL  *N*-(3-oxohexanoyl)-L-homoserine lactone	Bioluminescence[9]
Agrobacterium tumefaciens	OOHL  *N*-(3-oxooctanoyl)-L-homoserine lactone	Conjugal transfer of Ti plasmid[10]
Vibrio anguillarum	ODHL  *N*-(3-oxodecanoyl)-L-homoserine lactone	Unknown[11]

Table 1. Continued

Organism	Major signalling molecule	Phenotype(s)
Pseudomonas aeruginosa	OdDHL   *N*-(3-oxododecanoyl)-L-homoserine lactone	Virulence factors including alkaline protease, elastase, exotoxin A, haemolysin, neuraminidase[12]
Rhizobium leguminosarum	HtDeHL  *N*-(3*R*-hydroxy-7-*cis*-tetradecanoyl)-L- homoserine lactone	Expression of rhizosphere genes[13]
Escherichia coli	Unknown	Cell division[14]
Pseudomonas aureofaciens	Unknown	 Phenazine antibiotic[15]

this increasing AHL concentration enables the bacteria to monitor their own numbers indirectly, and only when enough bacteria are present does the molecule reach sufficient concentration to activate the relevant transcriptional activator. Given a system that requires the generation, reception and response to a chemical signal, one of the more surprising aspects of this method of gene regulation is the very small number of regulatory genes involved, and this simplicity will enable its exploitation (Fig. 1). It is likely that this molecular means of communication can be used both to detect the presence of pathogenic bacteria and, perhaps more importantly, to interfere with the pathogenic attack. In addition, the genes may be removed from their native background and used as a powerful genetic tool for coupling heterologous gene expression to cell density for biotechnological purposes.

Regulatory complexity

The AHL-dependent mechanism was first investigated in *Photobacterium fischeri*, in which the genes responsible for bioluminescence are expressed in response to increasing cell numbers. It was clear at an early stage that the underlying genetics of the regulatory circuit employed only two major genes[18], although it has since been realized that other genes play a minor, modulating role[19]. A single gene, *luxI*, is responsible for the generation of the signal, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), and a second gene, *luxR*, activates the expression of the remaining *lux* genes. Although the synthesis of AHL by LuxI and homologues had long been presumed to occur, AHL production by purified protein has only recently been demonstrated[20–22]. The *lux* system has become the point of reference for all subsequently described regulatory systems featuring homologues of these two genes, despite the fact that the genetic arrangement and regulation of the *I* and *R* genes in this system now appears to be somewhat atypical.

In *P. fischeri*, bioluminescence is the only phenotype shown to be subject to AHL-mediated control. It appears that the AHL is the main trigger for *lux*-gene expression, although modulation in response to other factors, particularly nutritional status, has been noted[23]. In *Erwinia carotovora*, the situation is a little more complicated, with the same signal, OHHL, being interpreted by the bacterium in two different ways. OHHL acts both as a command in the activation of carbapenem biosynthetic genes[5,6] and as only one of a number of inputs required before genes encoding plant-cell-wall-degrading enzymes are activated[7]. The bacterium ensures that before these exoenzyme virulence factors are produced, information is gathered not only on cell numbers, but also on the availability of substrate, as well as a wide range of other environmental factors[24].

Perhaps the most complex of the LuxI/R-type systems investigated to date is that employed by *Pseudomonas aeruginosa*. This human pathogen utilizes two transcriptional activators, LasR and RhlR (also called VsmR), and at least two different AHL-signalling

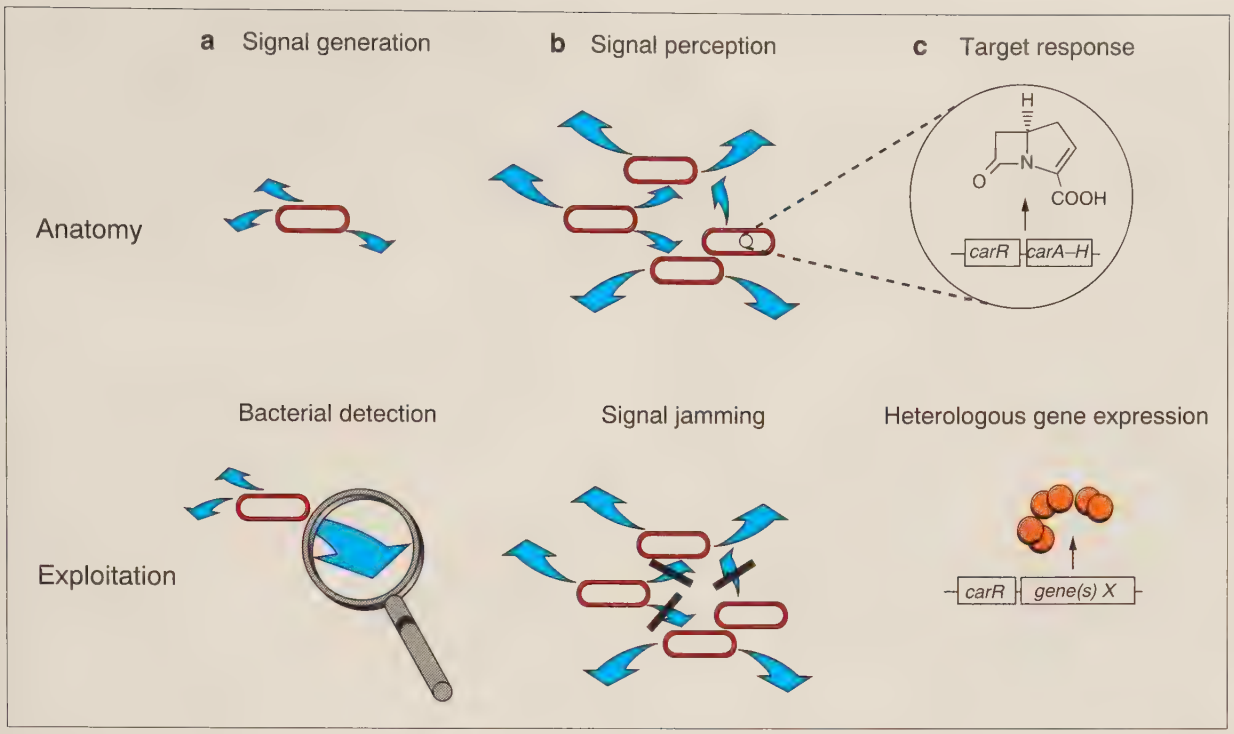


Figure 1

Anatomy and exploitation of bacterial *N*-acyl-homoserine-lactone (AHL) communication. (**a**) AHL is produced as a signal by the bacterium. Exploitation: the detection of the AHL by an external sensor allows the detection and enumeration of the bacteria. (**b**) Increasing concentrations of AHL are sensed and AHL-dependent functions are initiated. Exploitation: the use of AHL antagonists may prevent proper sensing and response. (**c**) The response requires the presence of a transcriptional regulator that activates target genes. In *Erwinia carotovora*, CarR causes expression of *carA–H* in response to OHHL, producing carbapenem antibiotic. Exploitation: placing heterologous genes under the control of the AHL-sensing system couples expression to an AHL signal.

molecules, OdDHL and BHL [*N*-(3-oxododecanoyl)-L-homoserine lactone and *N*-butanoyl-L-homoserine lactone, respectively] to activate expression of a large number of virulence factors (Table 1). Recent work has identified a regulatory cascade, with LasR and OdDHL activating expression of both RhlR and the stationary-phase sigma factor RpoS[1,25–27]. This work is at an early stage, and it is not yet clear which of the LasR–OdDHL and RhlR–BHL pairs activate which of the virulence factor genes known to be expressed in a cell-density-dependent manner. In addition, and in common with *E. carotovora*, there is also evidence for the influence of other physiological and environmental factors affecting expression of the virulence genes[26].

Eavesdropping on the signal

Despite the increasing regulatory complexity in the three examples above, a key aspect of each is the need for the populations to monitor their own demographics by the generation and perception of an AHL message. As a consequence of the release of AHL, the presence of bacteria may be detected indirectly using an appropriate AHL sensor. A method of this type, using the *luxR* gene product to detect OHHL and the remaining *lux* genes to report this detection, has been proposed for the enumeration of *P. aeruginosa* in pharmaceutical reagents[28]. An extension of this approach to detect other AHL-producing organisms is easy to envisage, and is reliant only upon the replacement of the relevant AHL-specific *luxR*-type (*R*) gene and its binding site upstream of the reporter gene. Several variations on this theme have now been reported[2,29].

The role of quorum-sensing in the virulence of plant and animal pathogens seems to be in coordinating the attack so that it occurs only when host-cell defences are most likely to be overwhelmed, and so rising AHL levels indicate rising preparedness for assault. Engineering host-cell defence genes so that they too are capable of responding to the AHL and can make a counter-attack is an intriguing possibility. Conceptually, this would be similar to the hypersensitive response seen in some plant infections[30], in which the detection of virulence factors by the plant leads to the expression of resistance genes leading to localized host-cell death to limit pathogen spread and the onset of disease.

Jamming the signal

AHL signalling systems play an important role in the control of virulence in some pathogenic bacteria. Disruption of this communication pathway offers an opportunity to prevent the bacteria from responding to the signal and thereby prevent proper expression of virulence factors. Interest has been growing in the possibility of using AHL antagonists as therapeutic agents to control infection. Potential methods for the

control of *P. aeruginosa* based on this approach have already been outlined[29].

Inhibitors may be identified by screening natural products or by the modification of naturally occurring AHLs. Recent studies have attempted to characterize the basis of the interaction between AHLs and their cognate LuxR-type (R) proteins[20,31] by testing the effects of a range of structural analogues on AHL-directed expression and native AHL binding involving LasR and LuxR, respectively. Several compounds can compete with the endogenous AHL for R-protein binding, but frequently these also serve as activators of AHL-driven expression. Although, in general, the best competitors were also the best inducers, several AHLs were able to compete with the endogenous OHHL sufficiently to cause a considerable net reduction in *lux*-gene expression. These studies have provided important groundwork that should assist in the design of synthetic therapeutic analogues. The next stage of development would be to identify molecules that essentially eliminate expression and that would bind the R protein irreversibly.

The observation that a variety of AHL analogues can activate the expression of AHL-dependent genes or inhibit the activity of the natural AHL has focused attention on the idea that interspecies communication may occur. This could represent anything from a means of acquiring information about the total population, allowing a response to competitors, potential associates or simply the prevailing conditions, to a method of direct competition if the AHL has a detrimental effect on other species.

The term 'alloinduction'[32] has been used to describe the activation of genes in one organism by coexisting bacteria. 'Alloinhibition' is used to describe the case where a signal from one organism interferes with the physiology of another to affect its ability to colonize a particular habitat. Alloinhibitory molecules would clearly be of interest in the wider control of AHL-dependent activities.

Studies of the ways in which AHLs are used in interspecies competition may be informative. The AHL produced by *Rhizobium leguminosarum* is interesting in this context as it seems to have a dual role, having been identified as an activator of gene expression and as a bacteriocin[13,33]. It has been suggested that this AHL may play a role in competition for root-nodule occupancy between *Rhizobium* strains in the rhizosphere; its bacteriocidal effect seems to be due to the fact that the signal can induce growth-inhibitory functions that result in an early onset of stationary phase in susceptible strains.

A recent study has shown that alloinhibition of bacteria by eukaryotic cells can occur and defines a new set of AHL analogues that may become important in this regard[34]. The marine macroalga *Delisea pulchra* produces a number of halogenated furanones, which are structurally similar to AHLs and have strong biological activity. These furanones have been shown to inhibit swarming motility in *Serratia liquefaciens*, a process known to be AHL-regulated[2]. This effect was not caused by a general interference with growth or metabolism but was specific to the signalling pathway. In the marine environment, furanone production probably helps *D. pulchra* to control its natural bacterial competitors. It would be surprising if this effect were unique, and so screening similar organisms for an ability to exert control over the ecology of bacterial species may provide a source of further interesting compounds.

Activating cryptic genes

Alloinduction and alloinhibition represent situations in which bacterial cells in a microbial community respond to signals generated by other species. It is even possible that some bacteria have functions that are expressed in response to AHLs, even though they do not themselves synthesize any AHL; in the absence of their AHL-producing partners, these genes would remain silent, and are referred to as cryptic. This phenomenon has already been demonstrated in some *Erwinia* strains, in which addition of OHHL causes the expression of otherwise silent genes responsible for the production of carbapenem (M. Holden, PhD thesis, University of Warwick, UK, 1996). Industrially, the addition of AHLs to microbial cultures in natural-product-screening procedures may induce novel functions of practical benefit[28].

AHL-regulated expression systems

AHL signalling systems offer a potentially simple switch for the induction of gene expression at a specific point in the growth phase. The expression window (Fig. 2) is opened when AHL reaches a critical concentration, but the natural induction point may be

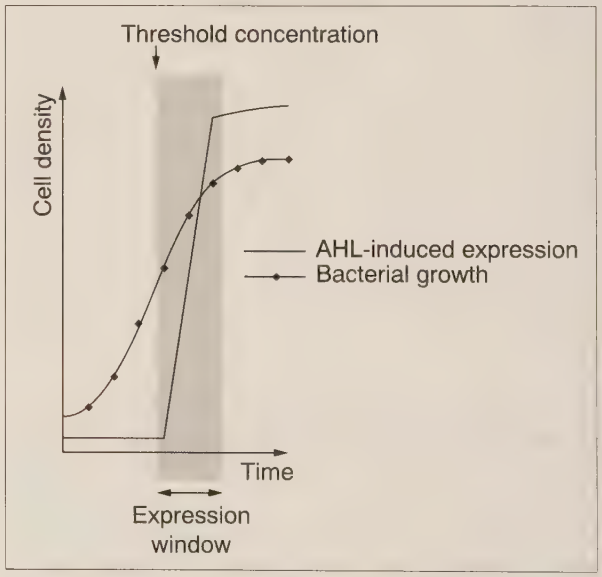


Figure 2
N-acyl-homoserine-lactone (AHL)-inducible window of gene expression. Expression of an AHL-regulated gene is coupled to cell density because induction only occurs when a threshold concentration of the AHL is reached; usually, this corresponds to a defined cell density. Addition of exogenous AHLs or analogues may shift the expression window by modifying the 'apparent' cell density.

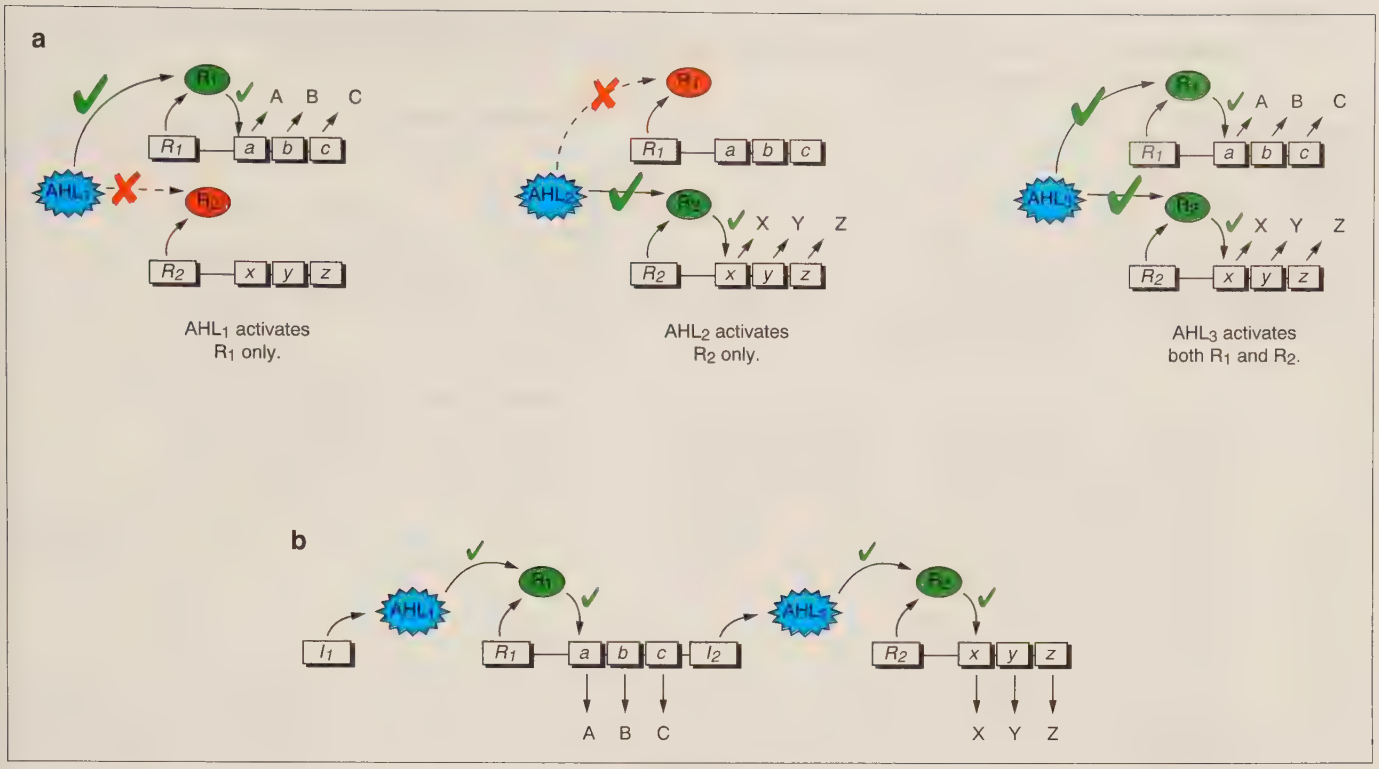


Figure 3

(**a**) Differential induction of gene expression. In a system in which two distinct sets of AHL-responsive genes are being expressed, controlled expression of each may be achieved in response to a particular AHL signal. Either set may be expressed singly if the specific AHL only induces one set of genes; conversely, both sets may be induced if an AHL representing a common signal is employed. The use of AHL antagonists may allow a further level of control, for example by allowing the shut-off of one branch at a particular point. (**b**) Sequential induction of gene expression. Expression of one set of genes may be separated temporally from that of another by coupling the synthesis of a second AHL synthase (encoded by I_2) to that of the first set of genes (*abc*) under the control of I_1. Production of the second AHL (AHL_2) and the subsequent induction of *xyz* will only occur after the production of proteins ABC has commenced.

shifted forward by the addition of exogenous AHL[28]. The expression window remains open until the AHL level falls, but if AHL synthesis is placed under positive-feedback control, as in the Lux system, levels will be maintained and the window will remain open. Placing expression of the required genes under the control of an *R* gene in the absence of a functional *luxI*-type gene, and using the addition of exogenous AHL to initiate expression, an extremely tight method of controlling gene expression can be produced. It has already been noted that such a method could be particularly beneficial for controlling the expression of deleterious genes by minimizing the harmful effects of a toxic product on the bacterial culture[35]. A more refined approach would use the manipulation of AHL levels to exert temporal control of gene expression, and this could enable easier downstream processing by separating product expression from that of other secondary metabolites. The development of this mechanism in an industrial context could therefore offer a number of significant benefits, and would be helped by the fact that it requires only two regulatory genes to enable the coordinate induction of multiple genes in response to an endogenous signal.

The family of R proteins are characterized by two binding domains; AHLs are thought to activate R proteins by binding to the N-terminal domain, while the C-terminal domain is involved in DNA binding upstream of the genes being transcriptionally controlled[36]. As the sequences of more R proteins become available, comparisons of the DNA-binding regions should help to elucidate the method(s) by which the R proteins, in both their active and inactive forms (with and without AHLs), function as activators or inhibitors of transcription. Utilizing this information should enable modification of the affinities of the R protein for AHLs and for its DNA binding site (by directed or random mutagenesis, or by the construction of chimeric proteins) and thereby allow further manipulation of the expression window.

It has been demonstrated that, while some R proteins are activated by a single specific AHL, for others, the response varies with different AHLs[31], and this is another facet of the AHL signalling systems that could be exploited. Combining the dedicated control exhibited by some AHLs and their R proteins with the broader-spectrum response offered by others could provide differential or sequential induction of different genes in response to different AHLs (Fig. 3). In *Chromobacterium violaceum*, for example, pigment production is induced by short-chain AHLs but can be repressed by the presence of long-chain AHLs[11]. Thus, it may be possible to use AHL analogues to inhibit the

Box 1. Potential biotechnological applications of AHLs and AHL-based systems.

Bacterial detection
Bacterial enumeration
Trigger for host-cell response
Activation of cryptic natural products
Control of bacterial virulence
Controlled expression of heterologous genes
Tight regulation of toxic gene products
Differential or sequential induction of gene expression

activation of R proteins and thereby halt the expression of the regulated genes at any given point. There is also evidence to suggest that, in the absence of any AHLs, some R proteins may block transcription and possibly compete (for the DNA target site) with other similar AHL-independent R proteins (A. Cox *et al.*, unpublished). Such a mechanism could provide further flexibility in a production-line approach to differential gene expression.

Through the presence of multicopy *R* genes, it is possible to bring about AHL-independent induction[5]. This indicates that the AHL dependence of R-protein function is not absolute, in some cases at least. The recent discovery of an AHL-independent R protein that causes constitutive gene expression rather than growth-phase-dependent induction (A. Cox *et al.*, unpublished) supports this idea. The flexibility offered by the variety of AHL signalling systems for regulating the induction of gene expression would be further enhanced by the facility to incorporate constitutive, growth-phase-independent expression where required.

Summary and future prospects

AHL-based signalling systems are being found in an increasing number of bacterial species. The genes controlled in this way seem to play important roles in determining the ways in which bacteria interact with each other and also with their eukaryotic hosts. The basic simplicity of these signalling pathways is appealing from a biotechnological perspective, but this apparent simplicity should not mask the fact that the ways in which they contribute to overall physiology in the cognate hosts may be subtle and complex.

Understanding of the underlying genetics and biochemistry of AHL-based signalling is developing rapidly, but the precise nature of the molecular interaction between signal and receptor, and between receptor and promoter sequence, remains to be elucidated. When achieved, this should help greatly in the design of AHL inhibitors and in the development of flexible promoter systems for the expression of heterologous genes.

As more and more information becomes available about AHL signalling systems, it seems likely that at least some of the potential biotechnological applications outlined in this review (summarized in Box 1) will proceed from the theoretically possible into practice.

Acknowledgments

Work in the authors' laboratory was supported by the BBSRC.

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Oligomer-chip technology

Jörg D. Hoheisel

Oligonucleotide microarrays have caught the attention of a wide scientific audience because of a collection of recent publications that demonstrate the technique's huge potential in a development that seems to be having similar consequences for molecular genetics as the arrival of the semiconductor had for electronics. From today's still rather confined basis, the methodology is bound to expand and diversify, radically transforming not only molecular genetics but also numerous other fields of application in medicine and pharmaceutical research.

The main emphasis of current genome analysis is still deciphering the basic sequence information of both the coding and noncoding regions of genomic DNA. However, technological development has already moved on to analytical procedures for the cellular effects and functional consequences of the DNA-encoded information. In recent years, DNA-chip technology has emerged as a prime candidate for the performance of many such analyses in molecular biology and medicine, particularly given the stimulus provided by the rapid increase in the amount of genetic information needing interpretation. This is because one of the main features of this technique is its high degree of parallelism, and the capacity to assay a large number of samples in a short time will make it almost indispensable. Several different methods for the creation of DNA arrays are being used, but the basic principle remains the same: hybridisation of an unknown sample to an ordered array of immobilised DNA molecules of known sequence produces a specific hybridisation pattern that can be analysed or compared to a given standard (Fig. 1). The array elements consist of either synthetic oligonucleotides or longer DNA fragments, usually PCR products or isolated cloned DNA.

The basic principle of using oligonucleotide arrays was first proposed in the late 1980s, when four groups independently developed the concept of determining a DNA sequence by hybridisation to a comprehensive set of oligonucleotides, such as all the possible 65 536 octamer sequences (sequencing by hybridisation, SBH)[1–4]. As indicated by the designation of the early scientific gatherings in this field[5], the initial aim was the establishment of SBH, in preference to any other application. Only with time did the true potential of oligomer arrays become clearer and the technical barriers to the (experimentally complex) process of SBH more obvious, shifting the emphasis to more immediately addressable applications.

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Basic aspects of oligonucleotide microarrays

Array production

Fabrication of the arrays is quite obviously of crucial importance to oligonucleotide-chip technology. Initial *in situ* synthesis techniques[7] would have been sufficient for the generation of comprehensive arrays, but for the combinatorial limitations of the channel system, which failed to synthesise entirely independent sequences at the individual grid positions. With the advent of a procedure that allowed the control of oligomer synthesis on the chip by photolithography[8,9], this shortcoming was resolved; in addition, a new dimension in the miniaturisation and complexity of the arrays unfolded.

Alternative production methods do exist, and these can produce features unobtainable by photolithography. Transferring individual DNAs onto a solid support[10], for example, seems to be a very tedious process, but it does permit a variation in the amount of material placed at each grid position; with modern high-throughput robotics, large-scale chip production by this method is well within our reach. Individual-transfer arrays possess an advantage over photolithographic arrays in their ability to be constructed with varying duplex stabilities of the oligonucleotides, merely by varying their concentration on the chip surface[11]; also, standard synthesis chemistry is well established for a number of nucleotide derivatives for which no light-inducible monomer equivalents are available. The critical drawback, however, is still the need for the external synthesis and storage of very many different oligonucleotides prior to actual chip production. Control of chip production by electrochemical patterning of surfaces using electrode arrays seems to be a viable alternative to photolithography in terms of size, speed and ease of synthesis[12]; whether it produces arrays of similar or even better quality remains to be seen.

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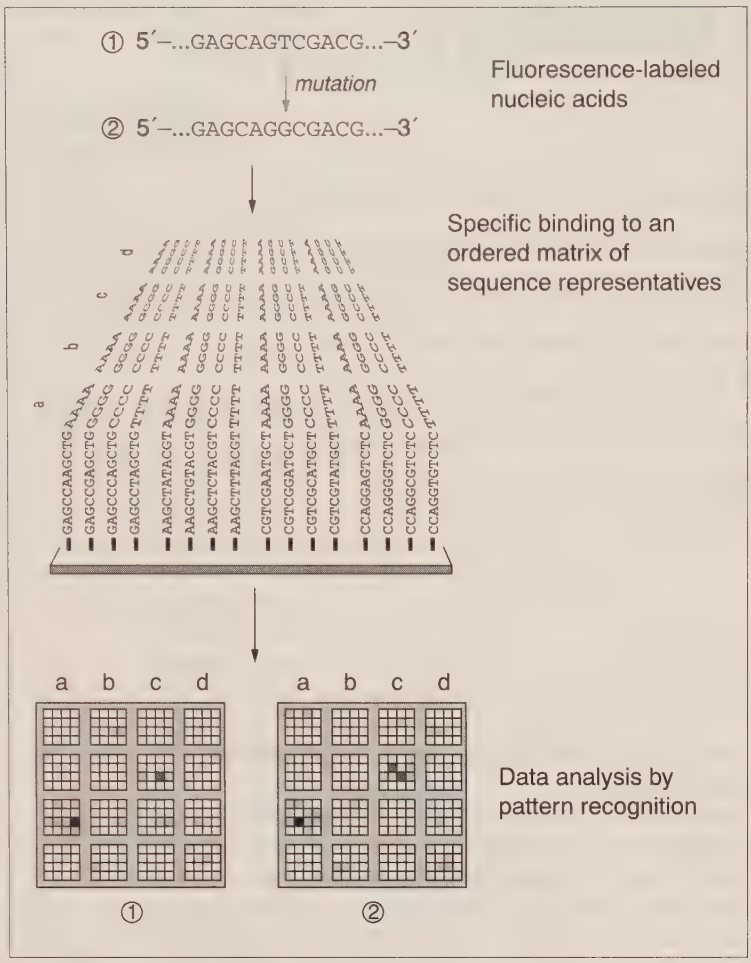

Figure 1

Basic principle of oligonucleotide-chip technology. Nucleic acids are exposed to an ordered grid of array-bound oligomer molecules. At each position, a distinct sequence is attached to the support medium, with the differences as small as a single base for the detection of polymorphisms, as displayed here. After hybridisation of the probe to chip molecules with a complementary sequence, analysis of the binding position is performed by pattern recognition. Evaluation of signal intensities is essential for the identification of the occurrence of mismatch hybridisation (modified from Ref. 6).

Even the current champion, photolithographic chip synthesis, has flaws, however, including relatively low yield obtained on the solid support[13]. This is at least one reason why a large degree of redundancy is required in actual analyses; although not necessarily an obstacle for some applications, this remains a problem for quantitative measurements. Work is ongoing to improve the efficiency of synthesis towards that achieved on standard support media[14,15].

Hybridisation

After production of the oligomer chip, the successful performance of target hybridisation is the most important issue of chip-based analyses. Many effects are still not completely understood, including the influence of the dangling ends of a bound molecule on the stability of the duplex[16] or, even more importantly, how much of a probe is really accessible to hybridisation; the relatively small amount of published data indicates that the latter issue, especially, is not yet resolved[17]. It is, however, possible that there is more data on this subject in the private domain that will not be published in the foreseeable future because it could give its owner a competitive edge. Fragmentation of the probe would be one way around the problem of intrastrand secondary and tertiary structure, which prevents binding to the arrayed oligomers. However, this would simultaneously cause another problem concerned with the labeling: usually, a base-specific fluorophor label is incorporated in the probe, but after fragmentation, there could be different amounts of label contained in the various, very-short pieces generated, due to, for example, enzymatic preferences. This could lead to a bias when comparative analyses are performed.

Probe concentration is another issue that strongly affects hybridisation; in fact, it is the one major reason why chips are needed for this sort of analysis. Because the amount of sample is limited in many potential applications, the relevant assay must be performed in small volumes, and thus on small surfaces, in order to obtain the probe concentrations necessary. A possibly very useful technique in this context is directing the probe flow by applying electric fields[18]. In addition, this technology allows for electronic control of the hybridisation stringency, thus accelerating the process.

Detection

Currently, there is a sort of monopoly in detection methodology. The use of a fluorescently labeled probe requires that detection is performed optically, but there are, nevertheless, different approaches to this that could prove significant. Rather than using a confocal system[19], thus blocking out all signal from outside a certain layer, excitation could be done by taking advantage of optical wave guides, for example, which cause excitation to take place only in the zone reached by the evanescent wave along the wave-guide material[20]. Thus, only label that is very close to the support, almost exclusively bound probe molecules, will produce a signal. However, other techniques for circumventing the complex and costly confocal system are possible. The most elegant would be direct detection of probe binding by electronic means. There is research being done in this direction[21], but the published results are, to date, not very convincing. Nevertheless, this procedure would really merge DNA-chip technology with electronics, and make a pocket-sized machine possible.

Applications

The range of applications for this technique is already wide and will probably extend still further. However, I should mention that, for some assays, the use of longer array molecules could be advantageous; one of these applications is transcriptional profiling (Fig. 2), a technique pioneered in the early 1990s on filters[22–24]. Total mRNA is isolated and hybridised to arrays that contain gene representatives, either as PCR products[25,26] or oligonucleotides[27]. Miniaturisation has led to the technique now being fit to deal with entire genomes at high levels of sensitivity.

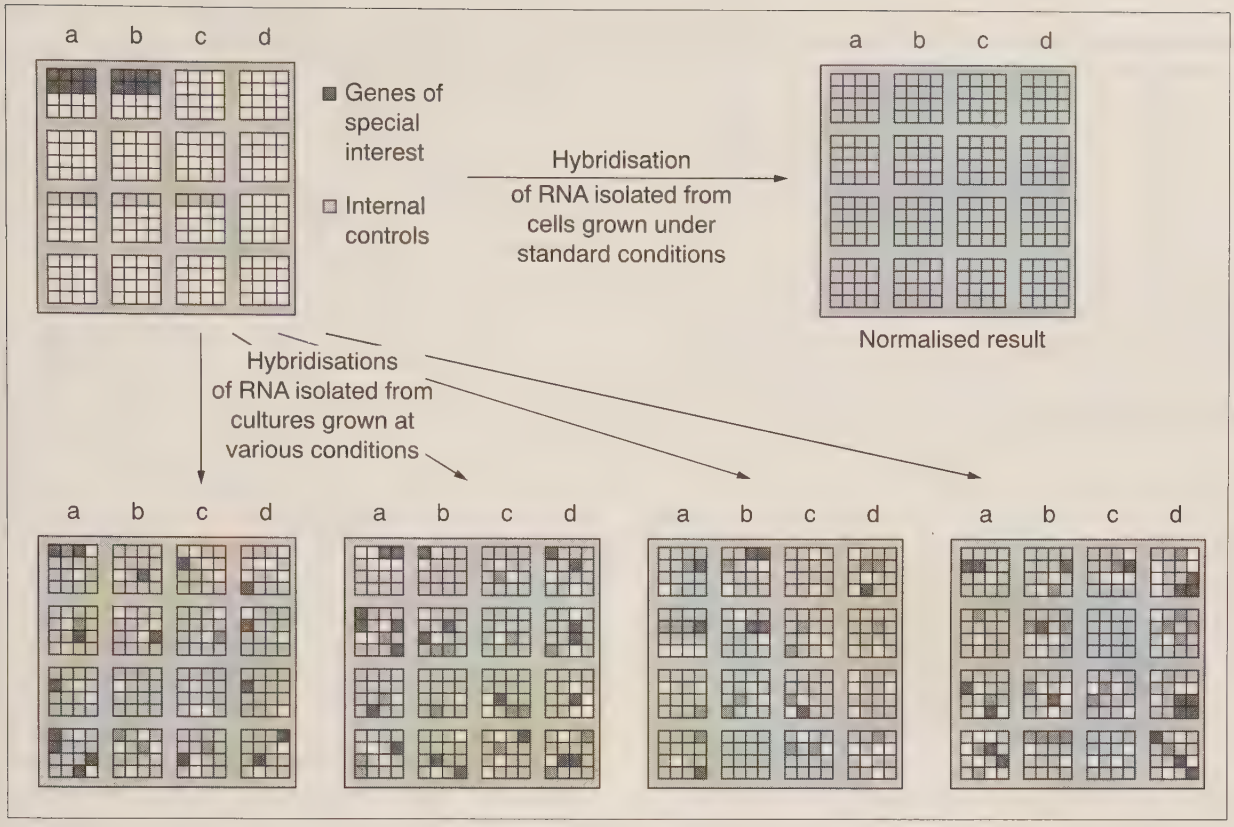

Figure 2

Parallel analysis of gene transcription. Total mRNA is isolated from cells and hybridised to a grid of molecules that represents (all) the genes of an organism. By analysing the binding positions and signal intensities, the transcription rate of each individual gene can be determined and compared with similar data generated with RNA from cells grown under different conditions. Using two dyes, two preparations could be mixed prior to hybridisation for immediate comparison.

Also currently at the forefront is the identification of mutations and polymorphisms[28–30]. By hybridising a sequence to a set of oligonucleotides containing all possible sequence deviations (Fig. 1), such analyses could be performed very quickly and also eventually lead to another sort of genetic map. While the physical mapping of individual cosmid or bacterial-artificial-chromosome (BAC) clones[31] seems an unlikely application, because it loses out on the issue of parallelism (and thus efficiency) compared with other hybridisation-based techniques[32], determination of the sequence-tagged-site (STS) content of larger genomic regions, such as radiation hybrids, could be worthwhile. Once one-tube PCR amplifications with very many pooled primer pairs can be performed, the presence of STSs in a given region could be checked by applying the result of such a PCR reaction to a chip that contains oligomer representatives for all the individual tag sequences (D. Cox, pers. commun.).

Other applications of oligonucleotide arrays range from studies in cell biology to chemical matters and include quantitative phenotypic analysis in a population of different (yeast) cells[33] (Fig. 3) and even highly parallel, and potentially extremely cheap, synthesis of primer molecules for subsequent use in molecular biology[14].

The most ambitious aim is still SBH, and a chip with 65 536 octamers, for instance, would also be the ultimate tool for many of the analyses listed above. The technique will probably never be used for *de novo* sequencing, however, because the read length is too limited in this sort of analysis; certain octamer sequences will occur more than once, thus creating many branching points within a given DNA fragment. However, the main sequencing task will only start after the human and other genomes have been completely sequenced. Then, individual patients, or distinct descendants of bacteria or other organisms, will be analysed in comparison with the sequence 'standard' that is in the database; for this application, the read-length of SBH is more than sufficient[34]. The last major technical obstacle was the need for equalising the duplex stabilities of all the different sequences. As there are ways to solve or get around this complication[35,36], no basic problem remains that could prevent SBH from becoming more than just a clever idea.

Peptide nucleic acids

More recently, a new class of derivatives, the peptide nucleic acids (PNAs; Fig. 4), have been developed[37]. Exhibiting many features that are identical, or at least very similar, to the behaviour of DNA oligonucleotides, such as the formation of a duplex with nucleic acids of complementary sequence, their application to oligomer-chip sensors might be advantageous for several reasons: their duplex stability greatly

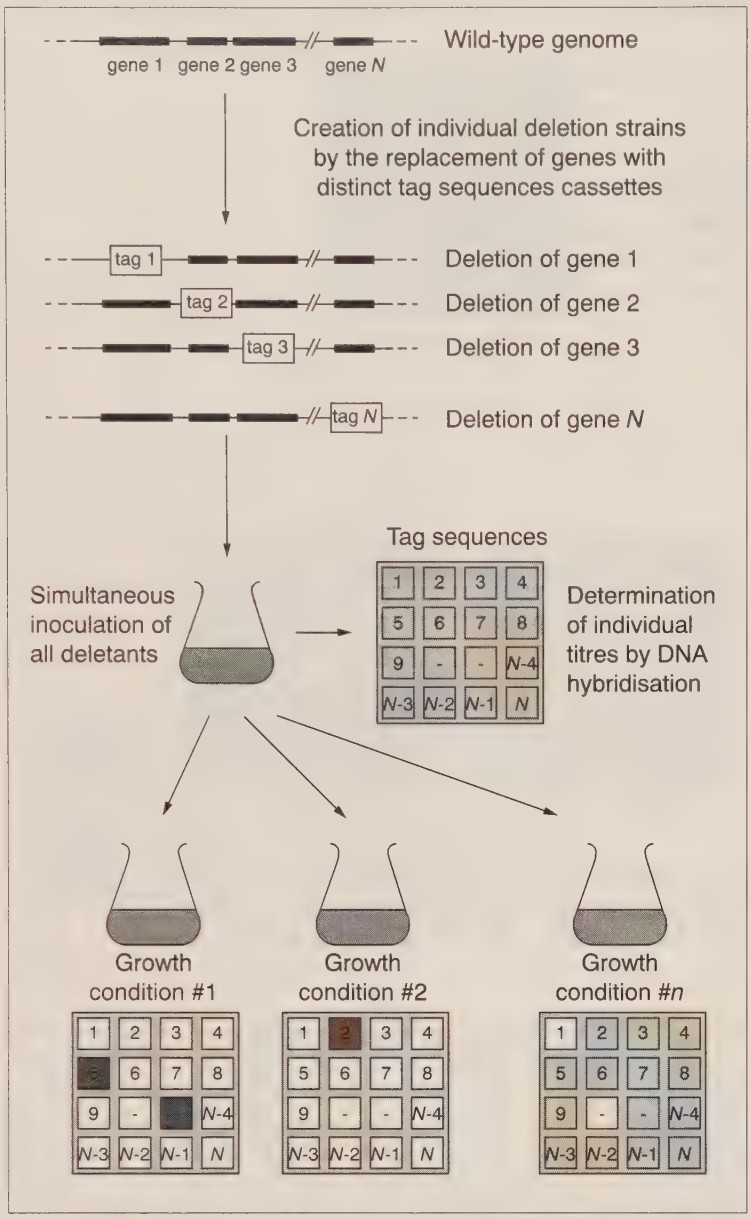

Figure 3

Phenotypic analysis in a population of different yeast cells. For functional analyses of gene activity, individual genes are replaced by DNA cassettes, each containing a distinct sequence of 20 to 40 bp. The deletants are all grown in one culture; at carefully designed growth conditions, individual deletions will be at an advantage or disadvantage, and this will lead to overgrowth or disappearance of the particular deletant strains. This is detected by hybridising DNA from the population of all cultured cells to a chip containing the tag sequences at specific locations.

exceeds that of equivalent DNA oligonucleotides; their binding to a complementary DNA strand is even more specific[38]; hybridisation takes place in a low-salt to possibly even no-salt buffer, and, because interstrand repulsion is not counteracted by ions, intramolecular double-strand formation of the DNA or RNA probe is disfavoured, making the entire probe sequence more accessible to the oligomers on the chip; and, less scientific but nonetheless important, the patent situation seems to be clear cut. Analyses with relatively small arrays of PNA oligomers has demonstrated the potential of these compounds as an alternative substrate for chip technology[39].

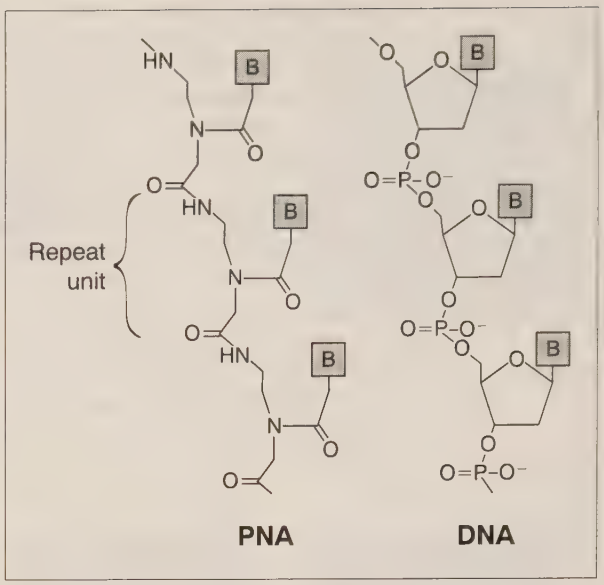

Figure 4

Comparison of the structures of PNA and DNA molecules. PNA consists of repeating units of *N*-(2-aminoethyl)-glycine linked by amino bonds. Bases (B) are attached by methylene–carbonyl linkages.

Scientific, commercial and medical applicability

There is no question that oligomer-chip technology will be put to productive and profitable use in science and many commercial applications, such as screening the molecular responses caused by the large numbers of compounds generated by pharmaceutical companies. Medical diagnosis in individual human beings is a different story, however, requiring the various analytical techniques to reach a standard sufficient to perform vital diagnoses reliably on individual patients. In practical terms of development and commercialisation, I wonder how regulative bodies such as the US Food and Drug Administration will set their accuracy requirements, given their different perspective. Considering the specificity required in practical uses of much simpler analytical tools such as primer oligonucleotides for PCR-based techniques, this is a crucial question, and a delicate one once it comes to commercial issues. Still, with the advances that have already been made being so enormous, the prospects for further improvement so substantial, and in view of related developments towards microfabricated structures for DNA analysis[40], I am convinced that a time will come when patients will give a drop of blood from their fingertip at a doctor's surgery, and shortly thereafter will be given a diagnosis based on chip-hybridisation data.

Acknowledgments

Work in the author's laboratory was funded by the Deutsche Forschungsgemeinschaft, the German Bundesforschungsministerium (BMBF) and the European Commission.

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Polyunsaturated fatty acids, part 2: Biotransformations and biotechnological applications

Iqbal Gill and Rao Valivety

The realization of the important biomedical roles of polyunsaturated fatty acids has led to the development of methods for obtaining and manipulating polyunsaturated lipids. Enzyme-mediated reactions have demonstrated unique advantages over chemical approaches and commercial lipase- and phospholipase-catalysed processes have been developed to address the mid- to high-value polyunsaturated-lipid market. Research over the past two decades has also highlighted the broad spectrum of bioactive products derived from the oxidation of polyunsaturated fatty acids. The potential of these compounds in the flavour, fragrance, pharmaceutical and fine-chemical arenas has encouraged the elaboration of biotransformation strategies based on isolated enzymes and whole cells.

In addition to the application of biotechnology to the primary provision of polyunsaturated lipids from natural sources (discussed in Part 1 last month), the second, and perhaps more interesting, area is the enzymatic and whole-cell-mediated conversion of polyunsaturated fatty acids (PUFAs; long-chain fatty acids containing two or more double bonds) to other bioactive compounds. Here, we examine developments in this domain, discussing first hydrolase-mediated reactions (in which the PUFA itself is unaffected but the overall lipid structure is altered to provide products with desirable nutritional, biomedical or surfactant attributes) and then bioconversions (in which the acid moiety is subjected to oxidative transformations that yield molecules with biological activities of relevance to the flavour, fragrance, pharmaceuticals and fine-chemicals industries).

Hydrolase-mediated biotransformations: speciality and structured lipids

The commercialization of hydrolase-mediated reactions in the food and pharmaceutical sectors[1] has rapidly led to the extension of this same technology to the PUFA arena. Enzymatic routes to polyunsaturated lipids are attractive alternatives to chemical processing for two main reasons. First, PUFAs are prone to oxidation, polymerization and isomerization reactions, which can occur all too readily during chemical operations and result in undesirable side products; such processes are almost eliminated under the mild conditions that characterize enzymatic conversions[1,2]. Second, structured lipids can be formed by utilizing the high specificity of enzymes, and the product profile can be engineered by the appropriate selection of biocatalysts and reaction conditions[1,2]. Lipase- and phospholipase-catalysed transformations are now in use in the food, nutraceutical (food and nutritional products ascribed with specific health-promoting biological activities) and biomedical arenas, where they are proving indispensable for upgrading and enriching PUFA oils and synthesizing specific polyunsaturated lipids.

Enriched glycerides, fatty acids and esters for food and biomedical applications can be obtained by the lipase-catalysed hydrolysis, alcoholysis, acidolysis, glycerolysis and transesterification of fish, plant-seed and microbial oils (Table 1). Reactions may be performed in solvents or in solvent-free mixtures, and PUFA levels can typically be elevated two- to eightfold, depending on the lipid substrate, biocatalyst and reaction employed[3–14]. Moreover, EPA, DHA, DPA and GLA (see Glossary) can be incorporated into seed oils, including those from soybean, groundnut, rapeseed and melon at levels approaching 40 mol% (mol% refers to the percentage of the total moles of fatty acid present)[15–18], thereby providing cheap polyunsaturate-

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PII: S0167-7799(97)01077-9

Table 1. Examples of lipase-catalysed enrichment of PUFAs from animal and plant lipids

Lipid source (substrate)	PUFAs[a]	$[PUFA]_{pre}$ (% w/w)[b]	$[PUFA]_{post}$ (% w/w)[c]	Lipase catalyst	Reaction (type)	PUFA product (enriched)	Ref.
Menhaden (oil)	EPA	21	40	*Pseudomonas* sp.	Hydrolysis	Gs (mostly MGs)	3
Cod liver (oil + FFAs)	EPA/DHA	9/13	22/34	*Rhizomucor miehei*	Acidolysis	Gs (mostly TGs)	4
Cod liver (oil)	EPA + DHA	20	40	*Pseudomonas* sp.	Alcoholysis	MGs + DGs + FFAs	5
Cod liver (oil + FFAs)	EPA + DHA	21	60	*Rhizomucor miehei*	Acidolysis	Gs (mostly TGs)	6
Tuna (oil)	DHA	25	53	*Candida cylindracea*	Hydrolysis	Gs (mostly DGs + TGs)	7
Tuna (oil)	EPA + DHA	38	58	*Geotrichium candidum*	Hydrolysis	Gs (mostly TGs)	8
Sand eel (oil)	DHA	8	48	*Rhizomucor miehei*	Esterification	FFAs	9
Sardine (oil + FFAs)	EPA/DHA	14/11	70/67	*Rhizomucor miehei*	Acidolysis	Gs (mostly TGs)	10
Borage (oil)	GLA	20	48	*Candida cylindracea*	Hydrolysis	Gs	11
Borage (FFAs)	GLA	20	93	*Rhizomucor miehei*	Esterification	FFAs	12
Evening primrose (FFAs)	GLA	9	75	*Rhizomucor miehei*	Esterification	FFAs	12
Biota orientalis (FFAs)	ETRA + ETA	14	61	*Candida cylindracea*	Esterification	FFAs	13
Mortierella alpina (oil)	AA	25	60	*Candida cylindracea*	Hydrolysis	Gs (mostly TGs)	14

[a]DHA + EPA refers to a substrate containing a mixture of the two PUFAs where both acids were enriched; the combined concentration is given. DHA/EPA indicates a substrate containing a mixture of the PUFAs, but where only one of the acids was concentrated; the individual concentrations are given.
[b]PUFA concentration prior to reaction.
[c]PUFA concentration after reaction.
Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ETA, eicosatrienoic acid; ETRA, eicosatetraenoic acid; FFAs, free fatty acids; Gs, glycerides (MGs, monoglycerides; DGs, diglycerides; TGs, triglycerides); GLA, γ-linolenic acid; PUFAs, polyunsaturated fatty acids.

fortified oils suitable for direct food applications. Interestingly, EPA glycerides containing both GLA and EPA are formed through the lipase-catalysed reaction of evening-primrose oil with EPA ethyl ester, opening the way to mixed ω-3–ω-6 lipids (see Glossary)[19].

Similar approaches may be applied to structured glycerides, which are desirable as components of nutritional supplements, infant formulas and therapeutic agents because of their superior assimilation and metabolism[20–26]. Mixed glycerides with PUFA levels of up to 95 mol% are obtainable by the esterification of glycerol with acid concentrates[20,21]. Alternatively, triglycerides (TGs) with 34–85 mol% of DHA and EPA may be formed by reacting the saturated compounds with the corresponding PUFAs or polyunsaturated esters[22–24], or by partial hydrolysis of PUFA-rich TGs followed by esterification with saturated fatty acids[25]. The extent to which such lipids can be engineered, in terms of the content and positional distribution of the acids, is illustrated by the synthesis of a human-milk-fat substitute by lipase-mediated transesterification of butter oil with PUFAs[26]. In addition, pure AA, DHA and EPA TGs are easily made by the direct esterification of glycerol, or the glycerolysis of PUFA esters[27,28], and high-purity monoglycerides with PUFA levels approaching 90 mol% can be made by the esterification of 1,2-isopropylidene-*sn*-glycerol with PUFAs[29,30].

In addition to the above nutritional lipids, PUFA-based emulsifiers (which combine surfactant and biological activities) can also be produced for nutraceutical, biomedical and drug-delivery usages[31–36]. For instance, propane-1,2-diol monoesters of DHA and EPA, which are 'healthy' (nutraceutical) food emulsifiers, are provided in high yield by lipase-mediated esterification[31]. Likewise, GLA and DHA react with 1-*O*-alkyl-β-D-glucopyranosides and polyglycerols in the presence of lipases to provide high yields of the 6-*O*- and 1-*O*-acylated products, respectively[32]. PUFA phospholipids, which display improved assimilation and bioavailability characteristics compared with esters or glycerides, are similarly accessible. Phospholipase A_2 performs the esterification of EPA and DHA with *sn*-2 lysophospholipids (1-*O*-acyl-*sn*-glycerol-3-phosphocholine and 1-*O*-acyl-*sn*-glycerol-3 phosphoethanolamine) to give pure *sn*-2-modified compounds[33–35]. Alternatively, 1,2-di-*O*-acyl-*sn*-glycerol-3-phosphocholines, with up to 80 mol% of EPA or DHA at the

Glossary	
***m:n*(ω-*x*) PUFA**	Trivial nomenclature: a PUFA containing *m* carbon atoms and *n* double bonds, in which the final double bond is located *x*-carbon atoms (inclusive) from the terminal (ω) carbon atom of the fatty-acid chain.
***m:n*$\Delta^{aZ,bE..zZ}$**	Systematic nomenclature: a PUFA containing *m* carbon atoms and *n* double bonds. The superscripts a*Z*, b*E*, etc. indicate the positions of the double bonds (carbon atoms numbered from the carboxyl end), and whether they are of *Z* (*cis*) or *E* (*trans*) configuration.
***sn*-Lipids**	Trivial nomenclature for glycerolipids, in which *sn* indicates the presence of a chiral glycerol skeleton numbered *sn*-1, *sn*-2, etc. In phospholipids, the phosphate moiety occupies the *sn*-3 position.
***sn*-*x*-Lysophospholipid**	A phospholipid which has been deacylated at the *sn*-*x* (*x* = 1 or 2) position.
AA	Arachidonic acid: 20:4(ω-6) or 20:4$\Delta^{5Z,8Z,11Z,14Z}$
ALA	α-Linolenic acid: 18:3(ω-3) or 18:3$\Delta^{9Z,12Z,15Z}$
DHA	Docosahexaenoic acid: 22:6(ω-3) or 22:6$\Delta^{4Z,7Z,10Z,13Z,16Z,19Z}$
DHGLA	Dihomo-γ-linolenic acid: 20:3(ω-6) or 22:3$\Delta^{8Z,11Z,14Z}$
DPA	Docosapentaenoic acid: 22:5(ω-3) or 22:5$\Delta^{7Z,10Z,13Z,16Z,19Z}$
EPA	Eicosapentaenoic acid: 20:5(ω-3) or 20:5$\Delta^{5Z,8Z,11Z,14Z,17Z}$
ETA	Eicosatrienoic acid: 20:3(ω-6) or 20:3$\Delta^{5Z,11Z,14Z}$
ETRA	Eicosatetraenoic acid: 20:4(ω-3) or 20:4$\Delta^{5Z,11Z,14Z,17Z}$
GLA	γ-Linolenic acid: 18:3(ω-6) or 18:3$\Delta^{6Z,9Z,12Z}$
LA	Linoleic acid: 18:2(ω-6) or 18:2$\Delta^{9Z,12Z}$

sn-1 position, are obtainable by lipase-catalysed transesterification with PUFAs[35,36].

There are also many examples of biomedical phospholipids containing LA that can be produced using phospholipase D (Ref. 37). Thus, 1-*O*-acyl-2-*O*-linoleoyl-*sn*-glycerol-3-phospholipids modified at the phosphate end can be produced by transesterification of the corresponding phosphatidylcholines[37]. For instance, conjugates with alcohols, hydroxyethers, nucleotides, amino acids, peptides, glycopeptides, ascorbic acid, kojic acid, aza-sugars, sialic acid, etc. can be prepared, for applications ranging from synthetic intermediates, antioxidants, enzyme inhibitors, anticancer drugs, antiviral agents, cell–cell-interaction mediators and drug-delivery systems. Although most of the conjugates synthesized to date contain LA, the use of phospholipase A_2 and lipases should allow the production of analogues containing other PUFAs at the *sn*-1 and/or *sn*-2 sites[37].

The commercial practicality of hydrolase-mediated bioconversions has been clearly demonstrated by the successful introduction of processes for producing purified PUFAs and speciality glycerides and phospholipids for use in foods, pharmaceuticals and cosmetics (Table 2). In addition, lipase-based processing can potentially provide access to bulk, refined, polyunsaturated feedstocks suitable for further biological transformation, an area of application that has been hindered by the high costs of PUFAs refined by current physicochemical procedures.

Oxidative and related biotransformations

The unique unsaturated functionality of PUFAs makes them excellent synthetic precursors, as shown by the spectrum of molecules derived from them in biological systems[48–60]. The bioconversions responsible are initiated by the oxidative activation of the double bonds, usually in the form of hydroperoxide or endoperoxide intermediates, which result from the action of lipoxygenases (LOXs) and cyclooxygenases (COXs), respectively. *In vivo* reaction cascades initiated by cyclases, lyases, dehydrases and peroxygenases transform hydroperoxy-PUFAs into flavour and aroma compounds, hormones, defence compounds and physiologically active products ranging from aldehydes, ketoacids and hydroxyacids to eicosanoids and oxylipins[48–60]. Endoperoxides give rise to cyclic eicosanoids and related compounds, which fulfill regulatory, antimicrobial, reproductive and ecological roles[48–57].

The variety and complexity of the structures that can be generated using oxidative enzymes, especially LOXs and COXs, makes these potentially powerful catalysts for synthetic applications. Flavouring and perfumery materials, oxygenated fatty acids and eicosanoids and their analogues are especially attractive

Table 2. Representative industrial applications of lipases to speciality PUFA lipid products.

PUFA product	Lipase catalyst	Substrate	Reaction	Applications	Ref.
FFA concentrates	*Chromobacterium viscosum*, *Pseudomonas fluorescens*	PUFA oils	Hydrolysis	Anticholesterolaemics, etc.	38
FFA concentrates	*Candida* sp.	Fish oils	Hydrolysis	Pharmaceuticals, nutraceuticals	39
DHA concentrate	*Candida* sp., *Penicillium* sp.	Fish oils	Hydrolysis	Pharmaceuticals, nutraceuticals	40
Glycerides	Thermostable lipase	PUFA esters + glycerol	Transesterification	Anti-inflammatories, etc.	41
Monoglycerides	Alkaline lipases	PUFA oils	Hydrolysis	Pharmaceuticals, nutraceuticals	42
sn-2-Diglycerides	(i) Phospholipase A_2 (ii) Phospholipase C	PUFAs + *sn*-2-lysophospholipids	(i) Esterification (ii) Hydrolysis	Anticoagulants, thrombolytics Nutraceuticals	43 43
Triglycerides	*Rhizomucor miehei*, *Rhizomucor javanicus*	PUFAs + triglycerides	Transesterification	Nutraceuticals	44
Triglycerides	Various lipases	PUFA lipids + PUFAs	Transesterification	Pharmaceuticals, nutraceuticals	45
Triglycerides	*Candida antartica*	PUFAs + glycerol	Esterification	Pharmaceuticals, nutraceuticals	46
sn-2-Phospholipids	*Pseudomonas cepacia*, *Humicola lanuginosa*	PUFAs + phospholipids	Transesterification	Anti-inflammatories, etc.	47

Abbreviations: DHA, dicosahexaenoic acid; FFA, free fatty acid; PUFAs, polyunsaturated fatty acids.

targets as a result of regulatory and/or synthetic considerations. Although most available knowledge on COXs relates to eicosanoid biochemistry, considerable information has been amassed on LOX-mediated reactions and a sizeable amount of research has addressed synthetic applications[61].

LOXs are ubiquitous in plants and animals, and typically convert the $\Delta^{1Z,4Z}$ diene moieties in PUFAs into $\Delta^{1Z,3E}$-4-hydroperoxides[58–61]. A range of lipoxygenases exist, which differ in their substrate specificity, stereo- and regiochemistry of hydroperoxide insertion, and secondary oxidase and lyase activities. For example, known plant LOXs can convert LA into the (8*R*)-$\Delta^{9Z,12Z}$-8-, (9*R*)- and (9*S*)-$\Delta^{10E,12Z}$-9-, and (13*R*)- and (13*S*)-$\Delta^{9Z,11E}$-13-hydroperoxides[58–61]. With higher PUFAs, the existence of more regioisomers, together with the formation of bis(hydroperoxides) correspondingly enlarges the product spectrum[58–61].

Hydroperoxide-derived aromas and flavours

One of the biological roles of LOX-generated hydroperoxides, especially in plants, is the generation of volatile feeding attractants, anti-infectives and hormones, as part of growth and maturation processes and wounding responses[56,57,62–64]. These compounds, whose biosynthesis usually involves the combined action of LOXs, hydroperoxide lyases, isomerases and reductases, can have potent and desirable sensory activities and are therefore of interest to the flavouring and perfumery industries. For instance, the distinctive fresh odours of leaves, fruits and vegetables and the flavours and aromas of many processed foodstuffs, such as olive oil and tea, arise from LA- and ALA-derived C_6- and/or C_9-aldehydes and alcohols[56,57,62–64] (Fig. 1).

The odours of edible mushrooms originate from aliphatic compounds including octenols, octenones, nonenols, nonenones, nonadienols and nonadienones, produced from the degradation of (10*S*)-hydroperoxides of LA and ALA (Fig. 1)[56,57,64]. Similarly, the smell of fresh seafood results from C_5, C_6, C_8 and C_9 volatiles formed by the action of ω-6 and ω-9 LOXs on AA, EPA and DHA[56,57,64]. Such volatiles are usually further transformed in plants by esterification, hydroxylation, cyclization and isomerization reactions to give esters, hydroxyalkenals, lactones, etc. as secondary bioactives[56,57,62–65]. In addition, lipoxygenases and peroxidases present in plants such as tea mediate the oxidation of carotenoids by PUFA hydroperoxides to produce odourants such as damascones, damascenones, ionols, ionones and alkenals[66].

Although the above substances can be extracted from natural sources, their scarcity and variations in

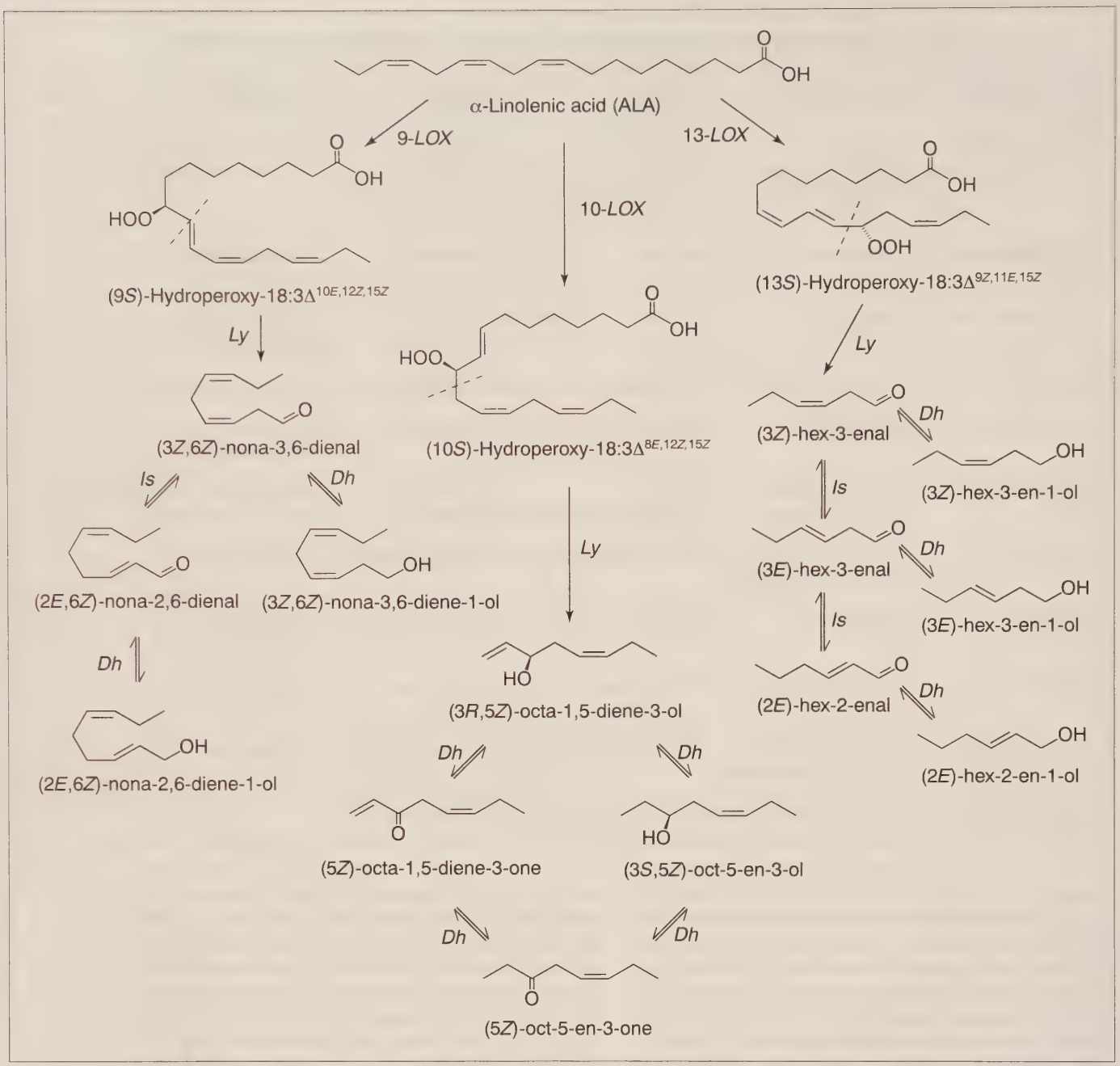
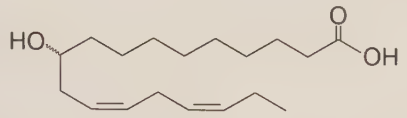


Figure 1

Lipoxygenase-mediated generation of C_6, C_8 and C_9 volatiles from α-linolenic acid. Abbreviations: *n*-LOX, *n*-lipoxygenase; *Dh*, dehydrogenase; *Is*, isomerase; *Ly*, hydroperoxide lyase.

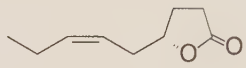
product quality have, together with a rising demand for 'natural' flavours and aromas, led to the elaboration of biotransformation routes. Despite the dearth of commercially available LOXs and lyases, and the resulting reliance on crude enzyme preparations, processes have been successfully developed for 'green note' (the characteristic fresh odours that characterize fruits, vegetables, leaves, etc.) and related volatiles. For example, the soybean LOX-catalysed conversion of ALA to (13*S*)-hydroperoxy-18:3$\Delta^{9Z,11E,15Z}$, followed by its cleavage by plant lyases and/or reduction by yeast (Fig. 1) is commercially used for producing (2*E*)- and (3*Z*)-hex-3-enals and (2*E*)- and (3*Z*)-hex-3-en-1-ols[67,68]. Similarly, the principal mushroom flavour components, oct-1-en-3-ol and oct-1-en-3-one, are accessible from LA using the ω-9 LOX and lyase activities of mushroom homogenates. Complex multisubstrate oxidations can also be implemented, as shown by the production of α- and β-ionone, 5,6-epoxy-β-ionone, (2*E*)-hexenal, (2*E*,4*E*)- and (2*E*,4*Z*)-decadienal from a mixture of PUFA and carotene feedstocks, via the combined action of LOXs, hydroperoxide lyases and peroxidases[69]. It is also interesting to note that C_6–C_9 volatiles, including (2*E*)-hex-2-enal, (5*S*)-non-2-en-4-olide (*S*-γ-nonenolide) and 2-pentylfuran are formed by the action of soybean LOX alone on LA, testifying to the multiple oxidase and lyase activities of these enzymes[70].

Box 1. Examples of bioactive products accessible from PUFAs via oxidative biotransformations

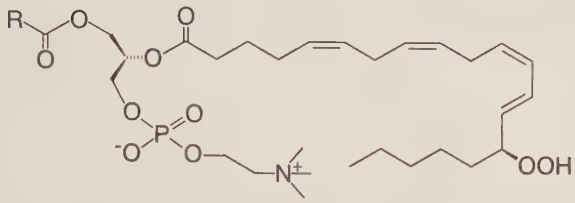


10-Hydroxy-18:2$\Delta^{12Z,15Z}$

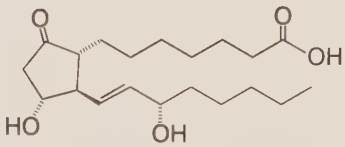
(4*R*,7*Z*)-Dec-7-en-4-olide



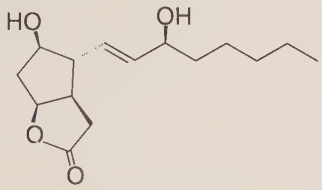
(5*R*,7*Z*)-Dec-7-en-5-olide



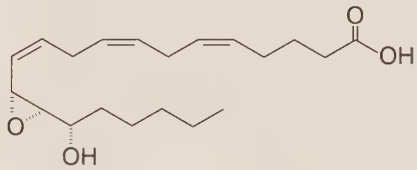
1-*O*-Acyl-2-*O*-(15'*S*-HPETE)-*sn*-glycerophosphocholine



Prostaglandin E_1 (PGE_1)

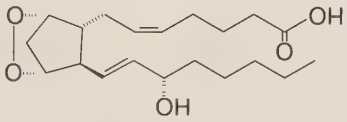


α-Pentanor $PGF_{2\alpha}$-γ-lactone

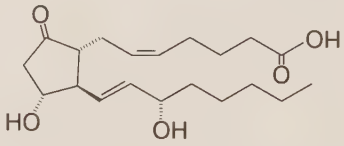


(13*R*,14 ,15*S*)-13,14-Epoxy-15-hydroxy-20:3$\Delta^{5Z,18Z,11Z}$

(5*Z*,14*Z*)-9-Oxo-(8,12-*cis*)-prosta-5,10,14-trienoic acid



Prostaglandin H_2 (PGH_2)



Prostaglandin E_2 (PGE_2)

It should be equally feasible to generate other volatiles from plant and fish oils using oxidase preparations from fruit, vegetable, mushroom and seafood processing residues[56,57,62–65]. The further characterization of LOXs and lyases from algae, higher plants and marine animals, together with progress on their isolation and cloning in microbes, should provide purified biocatalysts for synthetic applications[61,71–75]. However, although isolated enzymes are ideal for the synthesis of single odourants or simple mixtures, cell-culture technology is better suited to the reproduction of the complex sensory profiles that characterize natural products. The feasibility of this is shown by the transformation of LA and ALA to such compounds as hexanal, (2*E*)- and (3*Z*)-isomeric hexenals and hexen-1-ols, (2*E*)-hexen-1-yl acetate, (4*S*)-nonan-4-olide and (4*S*,6*Z*)-non-6-en-4-olide by plant cell cultures[76–78].

Hydroxyacids, oxylipins and eicosanoids

Hydroxy-PUFAs are valuable precursors to the flavour and fragrance lactones that occur in trace amounts in fruits, dairy and other foods, and as feedstocks for the manufacture of speciality polymers, resins, coatings, etc.[79] These acids occur in many plants, fungi and bacteria as PUFA-oxidation products, and, in addition, can be made via LOX-catalysed hydroperoxidations and whole-cell-mediated hydroxylations[61,80].

Nocardia spp., *Flavobacterium* spp. and *Acetobacterium* spp. transform LA, GLA and ALA to 10-hydroxy-derivatives of 18:1Δ^{12Z}, 18:2$\Delta^{6Z,12Z}$ and 18:2$\Delta^{12Z,15Z}$ (Box 1) respectively [81–84]. These compounds are analogues of riconoleic acid and can be transformed to sebacic acid and related monomers for application in plastics, lubricants, etc.[81–84] The ALA metabolite is also a direct precursor of the odourants (4*R*,6*Z*,9*Z*)-dodeca-6,9-dien-4-olide and (4*R*,6*Z*)-dodec-6-en-4-olide. Likewise, ALA can be converted to 9-hydroxy-18:3$\Delta^{10E,12Z,15Z}$, 12-hydroxy-18:3$\Delta^{9Z,13E,15Z}$ and 13-hydroxy-18:3$\Delta^{9Z,11E,11Z}$, which in turn are transformed into γ- and δ-lactones including (4*R*,7*Z*)-dec-7-en-4-olide (γ-jasmolactone, Box 1), (5*R*,7*Z*)-dec-7-en-5-olide (δ-jasmolactone, Box 1), (5*R*)- and

(5*S*)-decan-5-olide and (4*R*,6*Z*)-non-6-en-4-olide via fermentation with *Pichia* spp.[85,86] In a similar way, LA is oxidized in high yield by soybean LOX to (13*S*)-13-hydroperoxy-18:2$\Delta^{9Z,11E}$ (HPODE), and this is readily reduced to the corresponding hydroxy derivative, coriolic acid[87–90]. In addition to its natural occurrence in plant glycerides and its activities as an antifungal agent and biological mediator[91], coriolic acid is bioconverted by *Cladosporium* spp. and *Pichia* spp. to the flavourant (5*S*)-decan-5-olide (*S*-δ-decanolide)[92,93] and, also, its esters are potential replacements for ricinoleates[94].

Undoubtedly, the most impressive use of PUFA bio-oxidations is the synthesis of eicosanoid and oxylipin products. Thus, eicosanoid-pathway enzymes from mammals have been applied to the conversion of PUFAs to prostaglandins, which have many clinical uses[95]. Most effort has been directed towards prostaglandin-endoperoxide synthase (PGH synthase), which transforms AA to prostaglandin H_2 (PGH_2, Box 1), which in turn is converted to other 2-prostaglandins, prostacyclins and thromboxanes by isomerases, reductases, etc. Batch and continuous processes using enzyme preparations containing PGH-synthase and prostaglandin-E-isomerase activities have been developed for converting AA to prostaglandin E_2 (PGE_2, Box 1), a potent parturifacient (i.e. a substance that induces labour during pregnancy)[95]. Despite their obvious attraction, such processes are uneconomic for large-scale applications at present, owing to their low productivity and problems encountered with enzyme supply and biocatalyst stability.

The discovery that bacteria, fungi, algae, higher plants, insects and other invertebrates synthesize eicosanoids and oxylipins has raised the possibility of employing whole cells and isolated enzymes from these sources, thus avoiding the difficulties encountered with mammalian enzyme systems[52,95–100]. Thus, prostaglandin E_1 (PGE_1, Box 1) and other prostaglandins are produced by organisms including *Acinetobacter* spp., *Aspergillus* spp. and *Mortierella* spp. grown on GLA and DHGLA[96]. Similarly, various red, brown and green algae transform AA, DHA and EPA into the prostaglandin-like oxylipins[97]. *Saprolegnia* spp. oxidize AA to a mixture of hydroxylated oxylipins via such intermediates as (13*R*,14*R*,15*S*)-13,14-epoxy-15-hydroxy-20:3$\Delta^{5Z,8Z,11Z}$ (Box 1)[98]. Similarly, *Dipodascopsis* spp. convert AA to the prostacyclin metabolite α-pentanor PGF_{2a}-γ-lactone (Box 1)[99]. Oxylipins are especially prevalent in soft corals, and homogenates of gorgonians, such as *Plexaura* spp. elaborate AA to unusual oxylipins, including the prostaglandin-A analogue, (5*Z*,14*Z*)-9-oxo(8,12-*cis*)-prosta-5,10,14-trienoic acid (Box 1)[100]. Likewise, oxylipin-pathway enzymes isolated from plants may offer bioactives for application in agrochemicals, pharmaceuticals, etc.[61,101,102] Allene-oxide synthase and allene-oxide cyclase, which are key enzymes in jasmonic-acid biosynthesis, transform the (13*S*)-hydroperoxides of LA and ALA into epoxides, α- and β-ketols, lactones and cycloacids with anti-infective and growth-regulating activities[61,101]. Similarly, isomerases, peroxygenases and epoxygenases transform PUFA hydroperoxides into antifungal epoxyhydroxyacids and polyhydroxyacids[102].

Although no large-scale practical applications have, as yet, been demonstrated for enzymes associated with oxylipin and eicosanoid biosynthesis, the wide distribution of these biocatalysts should permit the elaboration of commercial biotransformation routes and so encourage product development.

Synthetic intermediates

LOX-generated products have also been utilized in the synthesis of selected biomedical targets[103–108]. For example, HPODE and the AA hydroperoxides, (5*S*,6*E*,8*E*,11*Z*,14*Z*)-hydroperoxyeicosa-6,11,14-trienoic acid [(5*S*)-HPETE] and (15*S*)-hydroperoxy-20:4$\Delta^{5Z,8E,11Z,13E}$ [(15*S*)-HPETE] are useful synthetic intermediates for PUFA metabolites associated with disease and aging processes[103–105]. Their introduction into the *sn*-2 site of 1-*O*-acyl-*sn*-glycerols and 1-*O*-acyl-*sn*-glycero-3-phosphocholines provides the corresponding 2-*O*-acylated lipids, for example 1-*O*-acyl-2-*O*-[(15′*S*)-HPETE]-*sn*-glycerol-3-phosphocholine (Box 1). These can in turn be converted into the corresponding phosphatidylserines, phosphatidylethanolamines or phospatidic acids using phospholipase D, or alternatively reduced to the hydroxyacid derivatives[103–105]. Likewise, (5*S*)-HPETE and the (5*S*,15*S*)-dihydroperoxide have been used as intermediates in the synthesis of leukotrienes and the toxic eicosanoid lipoxin A (Ref. 106). Interestingly, an elegant synthesis of the eicosanoid (5*S*,15*S*)-5,15-dihydroxy-20:4$\Delta^{6E,8Z,11Z,13E}$ methyl ester has been reported that employs substrate modification to effect a change in the positional specificity of soybean LOX[107]. Such reaction engineering, together with the availability of various LOXs, should provide access to a host of eicosanoid synthetic intermediates[108].

Conclusion

The realization of the biochemical roles of polyunsaturated fatty acids has focused commercial attention on PUFA-based nutraceuticals and therapeutics and on the provision of suitable synthetic frameworks for their production. The unique advantages and opportunities offered by the application of biotransformations in this field have been clearly recognized, and a host of bioconversion-derived products are now appearing, ranging from drugs for cardiovascular, dermal, neurological and immune-system disorders, to nutritional formulations and food additives.

In addition, PUFAs are versatile feedstocks for elaboration into a range of potent bioactives, demonstrated by their central roles in living systems. At present, the dearth of large-scale technologies for providing cheap, purified PUFAs has greatly restricted efforts in this direction. However, industrial interest in PUFA biomedicals should accelerate the development of biotechnological routes to high-grade ω-3 and ω-6 PUFAs. Major efforts are already under way to commercialize lipase-mediated processes, and this should,

with the development of microbial and plant-based production systems, soon make large quantities of PUFAs available for synthetic uses. Although research into oxidative bioconversions is in its infancy, the wealth of transformations and structures reported to date suggests practical routes for the production of oxygenated compounds that are difficult to obtain by chemical means. Indeed, once PUFA-production systems are in place, applications encompassing the production of aromas, flavours, eicosanoids, oxylipins, and chiral intermediates should be rapidly forthcoming.

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Exploring chromatography

Process Chromatography

by G. Sofer and L. Hagel, Academic Press, 1997. UK£80.00 hbk (ix + 387 pages) ISBN 0 12 654266 X

This is a very interesting and well-written book. Gail Sofer and Lars Hagel, both of Pharmacia Biotech, have written a comprehensive volume (it runs to almost 400 pages) in the form of a textbook rather than a collection of papers. Their work covers the many different aspects of process chromatography, often in considerable detail.

There is a very thorough 80-page chapter on purification design, optimization, and scale-up. This chapter covers all the well-known forms of chromatography – gel filtration, ion-exchange, reversed-phase, hydrophobic interaction and affinity. Parameters affecting performance are discussed and the ways that they can be controlled described. Points made are well-illustrated with chromatograms and secondary plots, many taken from research in the Pharmacia Labs. This chapter also cites over 100 references for further reading.

Another of the longer chapters deals with validation. As the authors correctly point out, 'validation should not be performed just because the regulators require it. Validation is good business and good science.' They go on to explain how to address issues such as the removal of endotoxins, assessment of ligand leakage, writing standard operating procedures, obtaining certificates of analysis for chromatography media, etc. The text is up-to-date and covers recent issues such as bovine-spongiform-encephalopathy contamination of bovine products.

There is also an extensive chapter on chromatographic theory, including a description of several mathematical equations that govern chromatographic behaviour – it would be a bit heavy-going for most biological scientists and would be of more interest to a biochemical engineer. The theory leads into a chapter describing the use of equations to model chromatographic processes; a disk is provided to be used in conjunction with this part of the text. For example, it is possible to enter different values for parameters such as the flow velocity, bed height, bead size and sample volume, and then to plot a predicted chromatogram from the modelling package. In my opinion, this disk would be of most interest to graduate students.

There are also several shorter chapters, covering a variety of topics. Some of these are excellent

and will be of general interest to scientists working in the biopharmaceutical industry, not just to chromatographers. For example, there are chapters on product sourcing (exploring the impact that different feedstocks have on regulatory issues), on the economics of running a chromatography process (discussing the contributory costs of items such as columns, media, eluants and labour) and on how to sanitise columns after use (recommending not only which cleaning solutions to use but also showing how effective they are for four different microorganisms). My favourite chapter of all was one of the shortest – entitled 'Production Scenarios', it explores the different process requirements for producing 100 g year^{-1}, 10 kg year^{-1}, and 500 kg year^{-1} amounts of a biomolecule by process chromatography.

In summary, this is a thorough, interesting and accurate book. It will be most useful as a reference book for scientists working in the biopharmaceutical industry and for graduate students in the fields of biochemical engineering or biotechnology, particularly those with an interest in an industrial career.

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Biotechnology invades chemistry

Biotechnological Innovations in Chemical Synthesis

edited by J. A. M. van Balken, Butterworth Heinemann, 1997. UK£35.00 pbk (x + 376 pages) ISBN 0 750 60561 8

The end of the 20th century is the beginning of the biotechnology era, and so it is time for not only biotechnologists but also chemical technologists in a wider sense to be ready to exploit its great potential. This means that chemists need to become more familiar with living (microbial) cells, their enzyme components and their potential catalytic applications in pure chemical syntheses. *Biotechnological Innovations in Chemical Synthesis* answers this need. In the series-editors' opinion, 'in this text, the potential of the biotechnological approach to chemical synthesis is described and the strategy and outcome of applying biotechnological processes and principles to the synthesis of organic products are explained.'

In the three introductory chapters, the authors outline the general potential of microorganisms and their enzymes as catalysts, and emphasize their benefits over synthetic-chemical methods. They deal in detail with the kinetics of microbial growth and metabolite production, and with their growth stoichiometry, discussing every important parameter from growth rate, through substrate yield and the maintenance coefficient, to the P:O ratio. Although this discussion of the quantitative behavior of microorganisms is invaluable, there is unfortunately not even a tangential reference to enzymes (for example, the basic kinetics of homogeneous and immobilized enzymatic reactions).

The real themes, discussed in the following seven chapters, start with a good selection of examples of classical industrial-fermentation processes, such as single-cell-protein production, microbial organic-acid syntheses (those of citric and itaconic acids) and microbial exopolysaccharides. The fourth and seventh chapters give purely fermentation-technological approaches.

Antibiotic production is introduced by a thorough discussion of their biosynthesis and diverse enzymatic modifications (which produce semisynthetic antibiotics), principally with reference to the β-lactams (penicillins and cephalosporins). This discussion is the first to give you the feeling that the potential of biotechnology is almost infinite.

Although every industrial fermentation process in these chapters is connected to a greater or lesser degree with chemical syntheses, the last two chapters best cover the main goal of the authors: to convey the idea that many chemical-synthesis problems can be solved by microbial or enzymatic biotransformations. The examples of amino acid fermentation, chemoenzymatic transformation (resolution of amino acids) and the highly specific biotransformations of lipids (mainly steroids) are covered here. This is very informative for organic chemists, as it shows the great range of possibilities that exist, and that could help in (stereo)specific group-introduction and -modification.

The merits of the book include the 77 self-assessment questions, which are problems to be solved, and the 'open doors to learn'. The self-assessment questions include rather sophisticated quantitative numerical examples to be calculated as well as brainstorming text-based questions (help is provided at the end of the book). The product-specific parts of this volume are mostly case studies with a strong engineering approach, and the authors always emphasize the technological and economic considerations.

This multiauthor work is a new volume in the *BIOTOL* series. As such, it is aimed at a broad range of the public: distance-learning students, those attending ordinary universities, and, not least, their tutors and teachers. This volume does serve as a valuable aid for anyone interested in the potential and current uses of biotechnology in chemical syntheses and can be highly recommended as a programmed-learning textbook.

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The life of mRNA

mRNA Metabolism and Post-transcriptional Gene Regulation

edited by J. B. Harford and D. R. Morris, Wiley Publishing, 1997. UK£80.00 hbk (xii + 354 pages) ISBN 0 471 14206 9

As is evident from the title of this book, the editors have undertaken the monumental task of covering the rapidly growing field of knowledge about the cellular events that happen to mRNA following transcription. The reader is eased in to the topic by concise but extensively referenced introductions to RNA structure and mRNA synthesis; a brief review of mRNA splicing is also provided, but the book's true focus is the large number of other post-transcriptional events that determine the fate of an mRNA molecule.

The organization of the book follows the 'life' of an mRNA molecule. The mRNA undergoes editing, polyadenylation, export, sorting, translation and, following a brief functional lifespan that is also subject to several regulatory events, degradation. The book concludes with descriptions of the post-transcriptional regulatory events that may contribute to some cancers and the many elegant mechanisms used by viral systems. These viral post-transcriptional control mechanisms may seem unique, but the current increase in our understanding of them indicates that many cellular genes may undergo both the well-characterized transcriptional regulation and also regulation during mRNA processing, translation or decay. The final chapters also serve to relate mRNA regulation to disease states; furthermore, most chapters strive to relate the mechanisms of post-transcriptional events to regulatory events occurring in a variety of eukaryotic organisms.

Most of the chapters provide the reader with diagrams of the particular topic, such as the multiple mRNA-decay pathways and viral translational-control mechanisms. Within a chapter, the format allows the reader quickly to locate the subtopic of interest, such as a particular initiation factor or a function of the poly(A)-binding protein. Most chapters provide multiple examples for the mechanism although, in some cases, the text concentrates on detailed descriptions of particular examples, such as the editing of the apobec-1 mRNA. While this may seem specialized, it provides great depth of information on the experimental basis of a mechanism, as well as being a primer for others interested in analysing a potential mode of regulation for a particular gene.

In order to provide a broad overview of the topic, some chapters cover areas not normally found in a review text, such as plant-mRNA decay and RNA sorting. In fact, this text is particularly good at filling in the gaps between standard review texts of translational control and mRNA turnover. Overall, the reader need not be an expert in the area to appreciate the information in this book, and it provides an excellent introduction or overview of the topic and a thorough description of the many different events that occur during the lifespan of an mRNA molecule.

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Assaying with antibodies

Principles and Practice of Immunoassay

edited by C. P. Price and D. J. Newman, Stockton Press, New York, 1997. UK£80.00 hbk (xi + 667 pages) ISBN 0 333 62504 8

Principles and Practice of Immunoassay is a series of monographs written by various experts and users of immunoassays with the purpose of providing an up-to-date view of this field. This includes topics that range from fundamental issues (e.g. the nature of antibody–antigen interactions) to more practical issues (e.g. the development of immunoassay reagents and immunoassay quality control), as well as an overview of common immunoassay formats and a summary of some newer assay technologies. Even though this is a second edition (the first edition having been published under the same name in 1991), this book stands entirely on its own and contains a large amount of new material that was not present in the original volume.

The first portion of this book (Chapters 1–7) consists of a discussion of antibody–antigen reactions and approaches to the development of antibodies or antibody-related reagents. For example, this section first examines the general structure of antibodies and antigens, and then takes a critical look at the importance of antibody–antigen interactions in immunoassay design. This is followed by a summary of various approaches for the preparation of polyclonal or monoclonal antibodies (including bifunctional

antibodies, alternative fusion protocols and the scale-up of antibody production) and the genetic engineering of antibodies. This section also includes chapters on the assessment of antibody–antigen kinetics (particularly by optical sensor systems), the selection of antibodies (based on, for example, affinity, specificity or stability) and the possible use of polymeric molecular imprints as alternatives to antibodies.

The next section of the book (Chapters 8–12) considers many other items of importance in the development and use of immunoassays. Topics discussed in this section include a summary of separation methods available for immunoassays, the optimization and description of analytical performance in immunoassays, and immunoassay quality control, standardization and data treatment. Throughout this section, there are many tools and techniques that should be valuable to those interested in the development of immunoassays for either in-house use or commercial applications.

The final portion of the book takes a look at many common or advanced types of immunoassays. This begins, in Chapters 13–18, with an overview of current methods for immunoassay automation and a review of immunoassay methods that use detection based on radiolabels, enzyme labels, fluorescent tags, chemiluminescent labels or light scattering. This is then followed by another section (Chapters 19–24) that deals with several newer and less-common immunoassay methods, such as techniques that make use of electrochemical detection, direct immunosensors, thin-film immunoassays and various types of integrated or microfabricated devices. The editors then complete the book by providing a glimpse into the issues and factors that they feel will be important in the future development of immunoassays.

This book is rich in information and presents a good overall picture of many topics of current interest in the field of immunoassays. As a result, it should have a wide appeal to an audience interested in the use, design or development of immunoassays.

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The how and the why

Recombinant Gene Expression Protocols

edited by Rocky S. Tuan, Humana Press, 1997. US$74.50 pbk (xx + 521 pages) ISBN 0 89603 333 3

Recombinant Gene Expression Protocols declares itself to have grown 'out of the need for a laboratory manual that provides the reader the background and rationale as well as the practical protocols for the preparation of "expression constructs" and their introduction into appropriate cells and/or organisms', but its success in realizing these goals is variable. In almost all of the chapters, the introduction is too brief, giving insufficient background. The protocols however, are generally good.

The book is divided into eight sections. Part I is an introduction by the editor, providing a brief outline of the book, and is written in a language closely resembling English. Part II (Expression in *E. coli*) is probably the best section of the book, providing sufficient background and detailed protocols for the uninitiated to perform the experiments described. It gives a clear explanation of the genetic foundation of *lac*-based cloning, an excellent introduction to cloning in λ, details of promoter and plasmid design, instructions for solving initiation problems and considerations for protein targeting to particular subcellular locations. Part III (Expression in Yeast) would have benefited greatly from chapters giving more background to yeast genetics. The chapter on *GAL4*-controlled expression stands out as achieving all the goals that the editor intended; other chapters discuss the use of different promoter systems, but without clearly highlighting the relative merits of each approach.

It is not obvious why Part IV (Viral Expression Systems) is separate from the mammalian-cell-expression section, as all the four chapters concern mammalian viruses, particularly in view of the fact that baculoviruses are discussed in the next section. This exemplifies the poor organization of the book. The chapters on vaccinia provide a good background and very detailed protocols, including a method for screening that avoids agarose overlays. The chapter on retroviruses has a welcome piece on the merits and disadvantages of this expression system. Part V (Expression in Nonmammalian Higher Eukaryotes) consists of the strange mixture of baculoviruses and *Xenopus*, leaving fruit flies and chickens to Part VII.

Part VI (Expression in Mammalian Cells) provides a mixture of excellent and far-from-excellent chapters, but is generally informative. When discussing COS cells, statements like 'the most convenient expression system' and 'the most widely used and convenient system for expression of a foreign gene is by introduction of DNA into COS-1 cells', without ever explaining why this is so, leave the reader feeling frustrated. There are then three chapters on transfection methods. Full marks to the authors of the chapter discussing calcium-phosphate and DEAE–dextran transformation for providing a rare comparison of methods. I was confused by a piece

on the magnetic isolation of somatic cell hybrids. This complicated method first requires the transfection of the two cell types to be fused with the neomycin gene and a cell-surface antigen gene. All this is to avoid the problem in conventional fusions, that failure to maintain HAT selection can result in reversion to the wild-type. My answer: maintain the selection.

The final two parts [Expression in Transgenic Organisms (surely they meant animals?) and Expression in Plants] would appear to be included only to provide something for those with a passing interest in these technologies, as there is far too little information to design or perform a transgenesis experiment. However, even if not practically useful, these chapters are clear, interesting and informative.

Apart from the poor overall organization and the general weakness of the second half of the book, the book does have something to offer. For those interested in recombinant gene expression in *E. coli* and/or yeast, the book could be very useful; those interested in gene expression in particular higher organisms might do better to go for a more specialized publication.

Finally, a whinge to the publishers: I hated the spiral-bound format of this book; my copy began to self-destruct after only a few hours. In the hard environment of the laboratory, I would not expect it to last long.

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To buy or not to buy?

Biotechnology for Non-specialists – A Handbook of Information Sources

edited by Lino Paula, EFB Task Group on Public Perceptions of Biotechnology, 1997. ECU 14 (xvi + 286 pages) ISBN 9 07611 001 8

The European public debate on biotechnology is more heated than ever, especially when food is the application of concern. Public-interest groups such as Greenpeace campaign strongly against biotechnology; companies try to market products such as genetically modified soy or maize.

In this context, the EFB Task Group on Public Perceptions of Biotechnology publishes *Biotechnology for Non-specialists*. This handbook aims to assist a wide range of people in locating the information that is available for the purpose of public communication on biotechnology, and, in a clear and concise manner, it draws a complete picture.

The book has four sections: the first describes most written information sources by title, reference, abstract and keywords, and is organised by country and also by subject, such as 'perceptions' and 'food and drink'; Section 2 provides an overview of key organisations; the third is a list of more than 70 Internet sites, hiding much information behind the addresses; Section four lists acronyms. Address and key-word indices complete the book.

As the publication is a reference book, it has to be complete and well organised. The degree of completeness is high – the only thing that is really missing is a list of the email addresses of the key organisations in Section 3. The arrangement of the book could, however, be improved. It is not clear, for example, why issues such as patenting and regulation did not deserve a separate section, for both are important topics in public debate. Fortunately, the clear key-word index helps to overcome this omission.

Is the book useful in the European biotechnology debate? In some situations it is, in others not. If you want to explain the current biotechnology-policy debate to the lay public, the handbook does not have much to offer, because it lists general information on biotechnology, while the current policy debate is becoming very scientific and case specific. Public-interest groups are currently very professional when they combat, for example, safety evaluations made by firms; their questions are different from those formulated six to eight years ago. The feeling then was that virus resistance might be a better thing than herbicide resistance. Now, very scientific and sophisticated questions are asked to assess, for example, the (dis)advantages of a specific virus-resistant crop. Is an antibiotic marker used? What is the exact genetic alteration? What about the resistance management of the food crop? In explaining this sort of debate, the book does not help – how can you explain to the lay public that some virus-resistant crops are more acceptable than others if the term 'virus resistant' still needs explanation?

Does this mean that the book is not useful? Not at all! Dr Bennet is right when he writes, in his foreword, that the need for such a handbook has never been greater. It is needed exactly at this time, when more and more biotechnological products are entering the market. Also, in the likely case that the debate continues, the public should know, at least roughly, what biotechnology is about. Everybody agrees that consumers have to be able to make an informed choice when they answer the ultimate question: to buy or not to buy?

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Novel vaccine technologies

At a recent conference*, two main themes emerged. Both concerned questions of balance – between induction of tolerance and immunity, and between the different components of the immune response to vaccination. Where the final balance of immune response to vaccination lies depends partly on the host and partly on the precise characteristics of the vaccination technique employed. Whilst the former is outside our control, we can select which technique to employ from the many that we have available to achieve the most-favourable host immune response in any given clinical situation. The conference considered the factors that might direct the selection of vaccination techniques to achieve any particular goal.

The first consideration in determining the characteristics of the host immune response must be the selection of a target antigen. G. Rook (Middlesex Hospital, London, UK) discussed the extraordinarily variable results obtained with one of the world's most famous vaccines, BCG. Part of the explanation for this phenomenon lies in the priming of the immune response by environmental mycobacteria. Furthermore, this priming may divert the desired T-helper-cell type-1 (Th1) response (with the consequent generation and maintenance of cytotoxic-T-cell responses) towards an unhelpful Th2 response, with the generation and maintenance of antibody responses. G. Rook has investigated the ability of SRL172, derived from *Mycobacterium vaccae*, to induce a Th2-to-Th1 switch in patients with late-stage melanoma. *In vitro* data using CD4 cells from these patients demonstrated spontaneous secretion of interleukin 2, a marker of a Th1 response. He also presented data suggesting that SRL172 may be able to down-regulate the Th2 response and so direct the immune response. A lively discussion was prompted by M. Gore (Royal Marsden Hospital, London, UK), who illustrated the difficulties, disappointments and hopes involved in delivering immunotherapy in the clinic rather than the laboratory. Happily, this heat produced light, in that delegates agreed on the importance of both collecting biological response data and looking for clinical responses, particularly when dealing with conditions, such as melanoma, in which the prognosis is highly variable and often unpredictable.

T. Monath (OraVax, Cambridge, MA, USA) could not be present at the meeting and so gave an 'attenuated' presentation by video. He described his group's work on vaccination against *Helicobacter pylori*, one of the most prevalent human infections. With up to 50% of adults in the industrialized world infected without immediate clinical problem, you may ask why it is worth vaccinating against this apparently innocent organism. The answer lies in the implication of *H. pylori* in the genesis of peptic ulceration, a rare tumour called MALT lymphoma of the stomach and, more tenuously, with gastric carcinoma. *H. pylori* produces an enzyme called urease and Monath's group have used subunits of urease to induce both serum and mucosal host responses. Protective immunization has been induced by oral, intranasal and parenteral routes, in that bacterial density is reduced and prophylaxis against reinfection is observed in animal models; phase-II trials of urease vaccination are due to start next year. The importance of inducing immunity to a number of different targets on the same organism, in order to minimize the effectiveness of escape mechanisms, was underlined by several speakers.

A much clearer link between infection and malignancy has been established between human papilloma viruses (HPV), genital warts and cervical cancer. Two HPV genes, E6 and E7, are consistently retained and expressed in approximately 90% of cervical cancers. N. Whittle (Cantab Pharmaceuticals, Cambridge, UK) and his group have investigated these genes as targets for specific immunotherapy. A phase-I study in patients with cervical cancer, employing recombinant vaccinia virus expressing modified HPV E6 and E7 genes, has demonstrated anti-HPV-E6/7 antibody responses in four out of eight patients. One of three patients tested also showed a positive cytotoxic-T-cell (CTL) response. M. Kast (Loyola University Medical Center, Maywood, IL, USA) said that studies are currently under way at the University of Leiden to assess whether antitumour CTL immunity against HPV E6/7 can be primed or boosted by peptide immunization of patients with residual cervical carcinoma following conventional treatment. He also presented data showing that peptide vaccination with viral epitopes can induce tolerance rather than immunity, illustrating that the method of delivery is the key to determining important characteristics of the immune response. He described, in outline, self-assembling virus-like-particle vaccines. The HPV L1 and L2 viral coat proteins self-assemble and can be made to contain the antigen of choice. This technique has several practical advantages – it targets the same tissue as the whole virus and also escapes endocytic vesicles. Early studies in animal models have shown promise in limiting tumour growth. Another technique was outlined by J. Shields (Cantab Pharmaceuticals). A disabled, infectious, single-cycle herpes-simplex virus (DISC HSV) has been created, in which an essential gene has been eliminated to create a virus that can infect a cell but only go through one round of replication, to produce noninfectious virus particles. The single round of replication allows for presentation of viral antigens via the class-I major histocompatibility complex, but eliminates the possibility of infection resulting from vaccination.

New antigen sources and delivery systems were discussed on the second day of the meeting. A technique that combines both a novel source of antigen and a novel delivery method was described by W. Swain (Genive, Madison, WI, USA). Here, DNA-coated gold particles are propelled by a high-pressure helium jet from a 'gene gun' into the host's epidermis. This technique exploits several advantages of targeting the epidermis such as accessibility, continuous exfoliation to increase safety, an excellent reservoir of immunocompetent cells and an ability to express transfected genes at high level.

The highly cost-effective use of plants as vaccine factories was described by W. Hamilton (Axis Genetics, Cambridge, UK). The amazing productivity of this system is revealed by the simple statistic that a single plant produces 1000 vaccine

**Novel Vaccine Technologies: New Advances in Technologies and Applications* was held at the National Heart and Lung Institute, London, UK, 3–4 July 1997.

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PII: S0167-7799(97)01130-X

doses. P. Laing (Peptide Therapeutics, Cambridge, UK) described the use of phage-encoded immunogenic peptides to mimic nonimmunogenic carbohydrates and hence raise an immune response. However, several delegates questioned this rational approach to vaccine design, in view of the emerging data concerning unexpected and unpredictable results of immunization. Another commented that he would 'trust to luck over insight any day of the week'. Perhaps, just like the immune response itself, this is another question of balance.

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Biotopic

Arbitration – a better way to resolve intellectual-property disputes?

Disputes are a fact of life. Many of those that arise in commercial dealings are resolved swiftly, often by making a telephone call or after an exchange of faxes. Others, however, are more intractable and may eventually result in litigation, with all its real and imagined horrors. Disputants will, quite rightly, go to great lengths to keep their problems out of the courts – but how many of them are aware that effective alternatives to litigation exist and are used every day? One such alternative is arbitration.

What is arbitration?

Like the elephant, arbitration is hard to define. It is probably easiest to establish what arbitration is by considering how it is similar to, and differs from, litigation in court. The similarities include the following.

(1) Litigation and arbitration are both means of resolving disputes, that is to say, getting to a final decision that disposes of the dispute.

(2) Both involve the imposition of a solution by a third party – a judge in the case of litigation, an arbitrator if arbitration is chosen.

(3) Both result in enforceable decisions.

(4) Both respect the principles of natural justice – that nobody should be a judge in their own cause and that both sides' arguments must be heard before a decision is reached.

The two processes, then, are structured to achieve the same result in the same way. There are, however, significant differences between litigation and arbitration, amongst the most important of which are the following.

(1) Arbitration requires an arbitration agreement

The fundamental distinction is that an arbitration cannot be started unless the parties to the dispute agree that there should be an arbitration. Court proceedings can be started whether or not there is any agreement between the parties that the dispute should be taken to court.

(2) Party autonomy

Because an arbitration owes its existence to a contract, and parties to a contract can agree anything that is not illegal, immoral or contrary to public policy, the parties to an arbitration have a great deal of freedom in the establishment and running of the arbitration. The parties thus have the power to do the following.

Choose who will decide the dispute

Anybody can be an arbitrator and arbitrators are often chosen because of their expertise in a particular discipline rather than because they are good lawyers. If a case goes to court rather than to arbitration, the judge will be chosen by the legal administration system without reference to the parties in dispute. Giving the parties power to appoint an arbitrator can be very useful in technical disputes – a great deal of time can be saved if they do not have to educate the arbitrator first.

Choose how many arbitrators there should be

It is usual to have one arbitrator only, as this makes for speed in decision making. There are, however, arguments in favour of more than one arbitrator on some occasions, particularly in international arbitrations if different cultures are involved, so that each party feels that its interests are fairly represented. The actual number, though, is a matter for agreement between the parties to the dispute.

Choose their procedure

The courts are more fettered by procedure (the rules to be followed to ensure the orderly conduct of proceedings) than are arbitrations. The parties to an arbitration have much greater power to decide on matters such as the taking of evidence, presentation of cases (how much will be in writing, how much given orally), the language of the proceedings and whether evidence will be given on oath. Often, in practice, these matters are dealt with by the choice of institutional rules, which set out procedural rules related to the conduct of an arbitration – use of the appropriate procedural rules is safer for those who are unused to arbitration, as there is less likelihood that anything will be left out of the procedure. There is, however, no reason in principle (and provided they agree) why the parties should not make up the rules as they go along.

Choose who will represent them

Unlike the courts, where (in general) only legally qualified counsel or the litigants themselves may conduct the case, arbitration takes a much freer view of representatives. The parties to an arbitration may be represented by anybody they choose, and many institutional rules provide expressly for this.

Choose whether or not to have a hearing

Many disputes referred to arbitration can be resolved by means of documents only. Each side provides a statement of its case and any written evidence (including witness statements) it wishes to adduce, and the arbitrator writes an award based solely on the documentation so produced.

Choose where any hearing will take place

There is no need to have a hearing in New York just because the laws of the State of New York govern the resolution of the dispute. If, for example, a patent dispute hinges on experimental evidence, it may be better to have the hearing in the lab in Cambridge instead of in a room on the tenth floor of an office building on Fifth Avenue.

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PII: S0167-7799(97)01126-8

Choose their timing to suit their mutual convenience

The timetable for litigation is set by the court and is invariably less flexible than the timetable that the parties to an arbitration can set themselves.

Choose their governing law

The parties may even decide that no law will govern, and that the arbitrator will decide using principles of fairness.

(3) Privacy and confidentiality

Court proceedings usually take place in public. Arbitration proceedings always happen in private – again, this is an aspect of the agreement between the parties. Intellectual-property disputes can involve a discussion of trade secrets and, although the courts offer safeguards against public disclosure, arbitration proceedings and the awards given by arbitrators are, by their very nature, confidential.

(4) Finality of awards

Arbitration awards are invariably final. Unlike court proceedings, where the loser may appeal as many times as there are superior courts to appeal to, there are very limited circumstances in which arbitration awards can be appealed.

Does arbitration or litigation take precedence?

The generally accepted rule is that, if court proceedings and an arbitration are started simultaneously in respect of the same dispute, the court proceedings will be held up until the arbitration has been disposed of. Furthermore, if litigation is started instead of arbitration when there is already an arbitration agreement in existence between the parties, the court will not allow the litigation to proceed – the parties will have to honour their agreement and go to arbitration. International treaties, notably the New York Convention of 1958[1], are in place to ensure that these rules are accepted world-wide.

As its full title suggests, the *New York Convention on the Recognition and Enforcement of Foreign Arbitral Awards* covers not only the international recognition of arbitration agreements but also the international enforcement of awards. One of the purposes of the New York Convention, to which 114 countries had acceded by 1 August 1997, was to simplify the enforcement of international arbitration awards and it has, to a large extent, achieved that objective.

The arbitration of intellectual-property disputes

A number of specialist bodies and procedures have been established to deal specifically with intellectual-property disputes.

The World Intellectual Property Organization's Arbitration and Mediation Center

The World Intellectual Property Organization, or WIPO, is an intergovernmental organization with its headquarters in Geneva. It is a specialized agency of the United Nations and had 165 member states in September 1997. WIPO's history dates back to 1883, when the *Paris Convention for the Protection of Industrial Property* was adopted, and to 1886, the date of adoption of the *Berne Convention for the Protection of Literary and Artistic Works*; WIPO itself was established in 1967[2]. It is responsible for the promotion of the protection of intellectual property throughout the world. Intellectual property is defined by the convention establishing WIPO as including the rights relating to: literary, artistic and scientific works; performances of performing artists, phonograms, and broadcasts; inventions in all fields of human endeavour; scientific discoveries; industrial designs; trade marks, service marks, commercial names and designations; protection against unfair competition; and all other rights resulting from intellectual activity in the industrial, scientific, literary or artistic fields.

In its own words,

> The WIPO Arbitration Center administers a number of procedures, which are alternatives to court litigation, for the resolution of international commercial disputes involving intellectual property. Those procedures operate within the legal framework which has grown out of international commercial arbitration and which is recognized in both national laws and international conventions.[3]

Four dispute-resolution procedures, including arbitration and expedited arbitration, are administered by WIPO. Any legally-recognized person, regardless of national affiliation, may refer a dispute to any of the procedures administered by the WIPO Arbitration Center. Given the commercial sensitivity of much intellectual-property work, the WIPO arbitration rules contain a separate and detailed section on confidentiality to clarify and reinforce the general principles and duties regarding confidentiality in arbitrations.

The Patents County Court Fast-track Arbitration Scheme

At the national level, the patents county court in England launched a Fast-track Arbitration Scheme in October 1996. The Fast-track Arbitration Scheme has its own rules, which are available from the Patents County Court in London.

Under these rules, disputes concerning intellectual property in its widest sense may be referred to arbitration by a single arbitrator when the arbitration agreement or an agreement supplemental to it provides that the rules apply, either expressly or by implication. The 'widest sense' of intellectual property includes the WIPO definition of intellectual property but presumably does not shut out any other subject matter if the administrator of the scheme can be convinced that the dispute should be arbitrated.

When to use arbitration and when not to

Anything that can be litigated can, in principle, be arbitrated, and probably more effectively, quickly and cheaply, especially if full advantage is taken of national laws and international conventions that exist to promote arbitration. There are, however, some instances when arbitration should not be used. Litigation should be considered instead of arbitration in any case in which publicity is desired, for instance where one of a number of alleged infringers of rights is being proceeded against with a view to discouraging infringements by others. Litigation, rather than arbitration, is also to be recommended in any case in which important or complex questions of law arise. Arbitration awards are binding only on the parties to the arbitration, whilst, in many jurisdictions, a court judgment creates a precedent that may be relied on when similar circumstances arise in the future (whether in litigation or arbitration).

Care should be taken in international disputes to ensure that the dispute can be resolved by arbitration in the relevant jurisdiction. Some legal systems do not allow patent disputes to be resolved by arbitration, arguing that a patent is a grant of

rights from the State, and that any disputes relating to the patent must therefore be resolved by the State's courts. Resolution will eventually be available, but it may be that the other side will have gained an immediate tactical (and therefore time) advantage. Time is invariably of the essence when dealing with the infringement of intellectual-property rights.

Nothing is perfect, and any arbitration has the potential to become not only more protracted but also more expensive than litigation. Not only do legal representatives require payment, but the arbitrators also command a fee and, if there is to be a hearing, accommodation in which to hold the hearing must be found and paid for. In litigation, the judge and the courtroom itself are invariably paid for by the State. Many jurisdictions are, however, addressing the issues of costs and over-lengthy arbitral proceedings. England has passed the Arbitration Act 1996, which imposes a positive duty on the arbitrator to avoid 'unnecessary delay or expense' and also on the parties, who 'shall do all things necessary for the proper and expeditious conduct of the arbitral proceedings'.

Competition policy

As an academic proposition, the protection of intellectual-property rights that confer a monopoly on their owner is, of course, at odds with the idea of freedom of competition, although most jurisdictions seem (rightly) to have little difficulty with a legal system that both grants patents and encourages competition. An increasing number of jurisdictions are becoming comfortable with the idea that anticompetitive issues can be resolved by arbitrators, even though, in earlier times, the determination of anticompetitive practices was thought to be the exclusive jurisdiction of the agency administering the competition policy. No jurisdiction has passed any legislation expressly making competition matters nonarbitrable.

In Europe, agreements 'which may affect trade between Member States [of the European Community] and which have as their object or effect the prevention, restriction or distortion of competition within the common market' are prohibited by Article 85(1) of the Treaty of Rome and any such agreements are void by virtue of Article 85(2). It is, however, possible to obtain exemption from the provisions of Article 85(1) from the European Commission under Article 85(3). An arbitrator cannot grant such an exemption, and an arbitrator who finds that Article 85 has been infringed must declare the infringing agreement void, which means that it never existed. What effect declaring a contract void has on the arbitration clause has long been a moot point. An arbitrator can rule on whether an agreement has been exempted from the provisions of Article 85 or whether it falls within a 'block exemption' [block exemption allows agreements that satisfy certain conditions (set out in the appropriate block exemption) to be exempted automatically from the provisions of Article 85 without the need for an application to the European Commission under Article 85(3) for specific exemption]. An arbitrator cannot, however, give a ruling that an agreement would qualify for an exemption if an exemption were applied for.

The European Commission requires that parties to an exempt agreement notify it of all arbitration awards relating to the agreement[4]. If an award takes the exemption beyond its permissible limits, the exemption may be withdrawn.

Model clauses and access to arbitrators

When drawing up a contract, as much attention should be paid to the dispute-resolution clause as to any other. It is too often too easy to put in a few words about dispute resolution moments before the contract is signed in the forlorn hope that the rest of the document is so well drafted that it can go into a filing cabinet, never to be seen again.

The best time to establish the mechanism for dealing with a potential dispute is when the deal is being done, when cooperation, rather than obstinacy, is the order of the day. There is (in theory, at least) no reason why an arbitration agreement cannot be entered into once the dispute has arisen, and such an approach allows the arbitration to be tailored precisely to the circumstances of the dispute. It is, however, usually much easier to agree a dispute-resolution procedure when the prospect of a dispute is so remote as to seem laughable than it is to try to put one in place when the parties cannot even agree on the time of day.

The various arbitral institutions, of which WIPO is but one, publish model dispute-resolution clauses geared to their own rules, which can be used when drawing up a contract. It is often worthwhile considering one of these clauses, as institutional rules are drawn up with the benefit of experience and will probably already make provision for circumstances as yet unforeseen by the parties. Institutional arbitration is, however, usually more expensive than ad hoc arbitration, in which the parties make up their own rules. If a dispute goes to an institutional arbitration, the parties will probably have to pay a contribution towards the upkeep of the institution as well as for the arbitrators. Institutions often charge fees calculated as a percentage of the amount in dispute.

The arbitral institutions maintain lists of accredited arbitrators, from whom an arbitrator with appropriate qualifications and experience can be chosen. A number of institutions will provide, on request, a list of names for consideration. Usually, the best way of getting access to accredited arbitrators is to include one of the institutional appointment clauses in the contract. Many of these appointment clauses provide for the appointment of an arbitrator by the institution concerned if the parties themselves cannot agree on an arbitrator.

Does arbitration have a place in intellectual-property disputes?

This is, in the end, a question that only the users of arbitration can answer. It is, however, worth repeating a few of the points made earlier. Arbitration allows:

the efficient determination of technical disputes by arbitrators who are well-versed in a particular industry, its processes and products;

confidentiality, both in relation to trade secrets and to avoid unnecessary publicity for a private dispute;

increased speed and reduced cost of resolution when compared to court proceedings;

control by the parties of the arbitration proceedings.

References

[Note: these documents should be easily located in any law library]

1 *The New York Convention on the Recognition and Enforcement of Foreign Arbitral Awards* (1958)
2 *Convention Establishing The World Intellectual Property Organization*, Stockholm (1967)
3 *The Services of the WIPO Arbitration Center* (1995) World Intellectual Property Organization, reference 445(E)
4 European Commission Regulation 17/62

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G-protein-coupled receptors in *Saccharomyces cerevisiae*: high-throughput screening assays for drug discovery

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G-protein-coupled receptors are an important class of therapeutic drug targets by virtue of their roles in the regulation of diverse cellular functions. Recent advances in the expression of heterologous G-protein-coupled receptors in the yeast *Saccharomyces cerevisiae* have led to the development of sensitive and selective assays of their ligand-induced activation. Implementation of this new technology in the high-throughput screening of compound libraries has enabled the discovery of novel ligands for the G-protein-coupled somatostatin receptor. This article describes the broad applicability of the technology and its use in drug discovery.

The G-protein-coupled receptors (GPCRs) constitute a large superfamily of transmembrane proteins that mediate cellular responses to diverse extracellular stimuli, including light, neurotransmitters, hormones and odorants[1]. Through selective ligand binding, the GPCRs discriminate between these multiple external signals. The GPCRs amplify and transduce the information inherent in ligand binding to the cell interior via interaction with intracellular heterotrimeric G-proteins. As a result, the G-protein complex undergoes guanine-nucleotide exchange and subunit dissociation in response to the agonist. After activation by agonist-bound GPCR, the GTP-bound α subunit and the $\beta\gamma$ complex are free to modulate the activity of a variety of effector proteins, including adenylyl cyclase, phospholipase Cβ, G-protein-gated Ca^{2+} and K^+ channels, and membrane-proximal elements of mitogen-activated protein kinase (MAP kinase) signal-transduction pathways[2]. Their integral role in cell signaling makes the GPCRs an important class of therapeutic targets for pharmaceutical research.

The significance of G-protein-coupled receptors

The GPCRs represent the targets for the majority of presently prescribed pharmaceutical drugs; ongoing pharmaceutical and biotechnological research continually adds new GPCRs to this list. Furthermore, functional-genomic efforts designed to survey the output of large-scale genome- and cDNA-sequencing projects have identified numerous orphan GPCRs, that is, receptor sequences recognised on the basis of similarity to known GPCRs whose cognate ligands are not known. Many of these orphan GPCRs will probably become the targets of future pharmaceuticals. The enormous diversity within the GPCR family presents the pharmaceutical researcher with the challenge of identifying those that are appropriate as targets for therapeutic intervention. Once target GPCRs have been identified, assays that sensitively and faithfully reflect the effect that chemical compounds have on target GPCRs must be developed. The assays should be sufficiently selective to identify compounds that possess limited cross-reactivity with related GPCR subtypes. In order to find 'lead' compounds that have the desired effect on the target GPCR, these assays are adapted for use in screening libraries of chemical compounds that are often enormous. By necessity, the assays must be high-throughput, that is, they must be rapid, convenient and flexible enough to handle large numbers of samples from diverse sources. Cell-based assays are useful in this context, because the target GPCR must perform most aspects of the functions it would carry out in its native context, including assembly into the plasma membrane in a proper ligand-binding configuration and transduction of the ligand-binding signal to the cell interior. A variety of cell-based systems have been used to express GPCRs and may be employed in the development of

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PII: S0167-7799(97)01119-0

Box 1. Cell-based high-throughput-screening assays for GPCR ligands

High-throughput screening (HTS) is the process by which large numbers of chemical compounds are rapidly tested for their ability to modify the properties of a selected biological target[3]. Well-designed HTS assays permit the widest possible array of chemical structures to be tested. These structures may be obtained from a variety of sources, including chemical files, combinatorial libraries, natural-product extracts and analogs synthesised during the course of structure–activity relationship programs, to maximise the likelihood of identifying suitable 'lead' compounds that possess desirable properties. The leads can then be modified and improved by medicinal chemists, leading to the eventual pharmaceutical drug. A variety of cell-based methods have been used for expression of the GPCRs[4]. In order to be suitable for use in HTS for GPCR ligands, cell-based assays should have the following properties.

(1) **Ligand selectivity:** the assay must accurately reflect the ligand-binding properties of the target GPCR expressed in its native context. This requirement must be stringently applied to microbial systems, including yeast and bacterial expression systems, in which substantial differences in membrane properties may lead to altered ligand-binding properties. Tissue-culture, cell-based screening systems closely mimic the native environment of the GPCRs but are limited by the presence of contaminating GPCRs that can confuse the analysis of ligand binding.

(2) **Convenience:** productivity in the HTS laboratory is greatly enhanced by limiting the efforts devoted to setting and keeping up the assay systems. Yeast-based HTS systems are easily maintained, requiring only simple sterile facilities and inexpensive microbial growth media and conditions. Stable transformants that reproducibly express GPCRs are easily and rapidly constructed. In standard practice, large cultures of microbial cells expressing GPCRs may be produced, validated and frozen well in advance; these frozen inocula may be thawed and used as needed. By contrast, the production of permanent tissue-culture cell lines of mammalian cells for expressing GPCRs can be a difficult and lengthy process, requires substantial time and effort for culture maintenance and uses expensive and complex growth media. The long generation time of frog melanophore cells, for example, and the use of transiently transfected cells would impose a considerable additional maintenance burden on an HTS lab[5].

(3) **Automation:** automatable HTS assays must be adaptable to robotic pipetting and assay measuring technologies, and the output must be readily adaptable to computer-based data-handling formats. The liquid-format yeast-GPCR-screening technology is readily adaptable to automation, requiring only two pipetting steps for the assay to be set up and run; assay output is easily measured in a conventional microtiter plate reader and the results can be written directly into a spreadsheet program. Functional assays of second-messenger accumulation in response to GPCR activation in tissue-culture cell assays are relatively difficult to automate, because they often require cell lysis or permeabilisation steps and additional manipulations in order to read the assay output.

(4) **Solvent compatibility:** the compounds found in most chemical files are dissolved in organic solvents such as dimethylsulfoxide (DMSO) or methanol, and so HTS assays must be able to withstand the effects of moderate concentrations of these solvents. Microorganism-based HTS assays are tolerant of the direct application of absolute DMSO or methanol to the agar plate and can withstand more than 2% with no loss of screening efficiency. Owing to the presence of a tough cell wall that is, nevertheless, permeable to even moderately high molecular weight compounds, yeast-based HTS assays can also withstand direct application of crude natural-product extracts. Many tissue-culture cells are sensitive to the toxic components and degradative enzymes occasionally present in natural products extracts, as well as to even small amounts of organic solvents.

high-throughput screening (HTS) assays (Box 1). This review will focus on recent developments in the expression of heterologous GPCRs in the yeast *Saccharomyces cerevisiae*, which promise significant advantages in the development and implementation of HTS assays.

GPCR-mediated signal transduction in the yeast *S. cerevisiae*

Many of the elements of GPCR-mediated signal-transduction pathways in mammalian cells closely resemble comparable pathways in *S. cerevisiae* (shown in Fig. 1a). Haploid yeast cells employ GPCRs to signal the beginning of the mating process[6]. Secreted mating-type-specific peptide pheromones are detected by cells of the opposite mating type through binding to G-protein-coupled mating-pheromone receptors (i.e. mating-type-**a** cells produce the **a** factor and express the α-factor receptor, Ste2; mating-type α cells secrete the α factor and express the **a**-factor receptor, Ste3). The pheromone-bound receptors induce guanine-nucleotide exchange by Gpa1, the α-subunit of the heterotrimeric G protein. Gpa1 dissociates from the complex of β (Ste4) and γ (Ste18) subunits, which in turn activate downstream elements in the pheromone-response pathway [for example, these include components of a MAP-kinase cascade that includes Ste20 (a p21-activated-protein-kinase homolog), Ste7 (MAP-kinase kinase or MEK), Ste11 (MEK kinase) and the MAP kinases Fus3 and Kss1[7]. Activation of the pathway results in cell-cycle arrest, transcriptional induction of pheromone-responsive genes and acquisition of cell structures related to the mating process. Many of the elements of the mating-signal-transduction pathway may be functionally replaced to varying degrees by their heterologous counterparts[8,9], allowing them to be studied in isolation from contaminating subtypes. Indeed, the striking resemblance between the components of the yeast pheromone-response pathway and mammalian GPCR-signaling systems suggested that yeast genetic approaches were likely to be useful for structure–function studies. Furthermore, yeast expression provides functional assays with high selectivity, conveniently measurable assay end-points and amenability to automated assay formats. These advantages make possible the development and implementation of mechanism-based HTS assays for the GPCRs, G-proteins and downstream signal-transduction proteins.

Heterologous GPCR expression in yeast

S. cerevisiae and other fungal cells have been used to express heterologous GPCRs for functional and structural studies (Box 2)[10–22]. In each case, ligand-binding

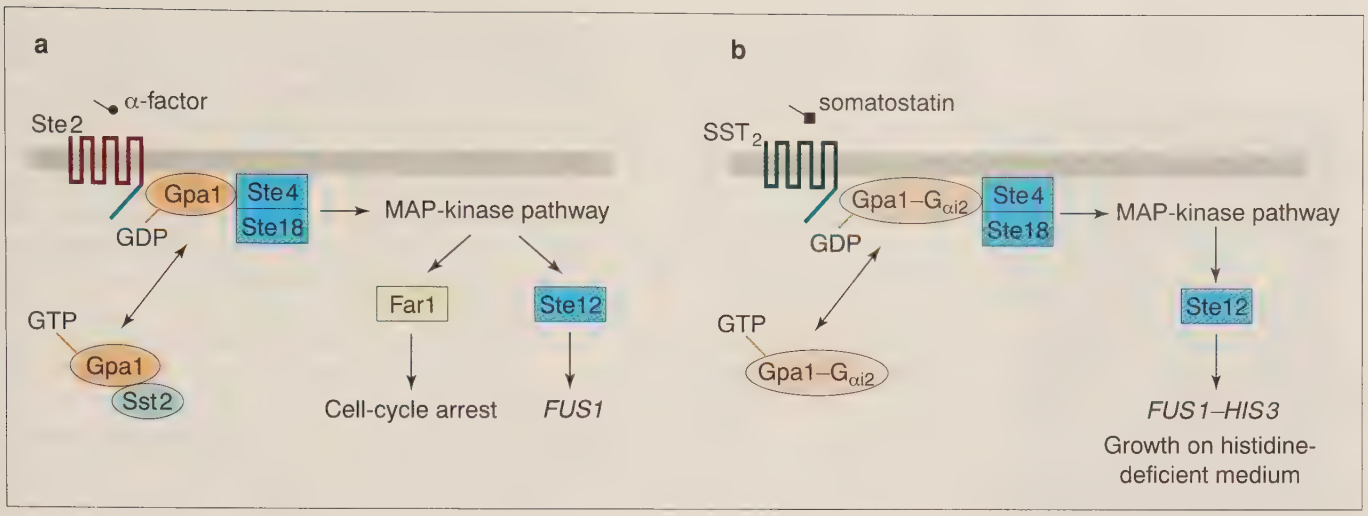

Figure 1

Genetic modification of the *Saccharomyces cerevisiae* mating-signal-transduction pathway permits agonist-induced growth on selective medium. (**a**) The mating-signal-transduction pathway of wild-type haploid **a**-mating type (MAT**a**) yeast cells is stimulated by the binding of the soluble tridecapeptide pheromone, α-factor, to the α-factor receptor, Ste2. The agonist-bound receptor catalyses the exchange of GDP for GTP bound to Gpa1, the α-subunit of the heterotrimeric G protein. Gpa1 dissociates from the complex of β (Ste4) and γ (Ste18) subunits, leaving it free to activate membrane-proximal elements of a mitogen-associated protein (MAP)-kinase pathway. The distal MAP kinase, Fus3, activates Far1, resulting in cell-cycle arrest; MAP kinase also activates the transcriptional activating factor Ste12, inducing the expression of proteins involved in the mating process and karyogamy, including *FUS1*. The yeast regulator of G-protein signaling, Sst2, reverses the activation of the mating pathway by enhancing the GTPase activity of Gpa1; after GTP hydrolysis, GDP-bound Gpa1 reassociates with the βγ complex, inactivating the mating pathway. (**b**) In genetically modified MAT**a** cells, genes encoding the yeast G-protein-coupled receptor (*STE2*) and G_α protein (*GPA1*) were deleted and replaced by plasmid-borne SST_2 receptor and Gpa1–$G_{\alpha i2}$ expression constructs. Somatostatin binding activates its expressed receptor, causing it to stimulate intracellular Gpa1–$G_{\alpha i2}$ and, hence, downstream elements of the mating-signal-transduction pathway. Deletion of *FAR1* prevents cell-cycle arrest; somatostatin-induced expression of a *FUS1–HIS3* reporter gene provides the His3 enzyme necessary for histidine biosynthesis, permitting growth on medium lacking histidine. Deletion of the *SST2* gene resulted in increased sensitivity to agonists acting on the heterologous GPCR, owing to low residual Gpa1-GTPase activity in the absence of Sst2. The deletion of *STE2* also had the effect of increasing the sensitivity to agonists.

Box 2. Fungal host cells for the expression of GPCRs

The cells of the fungal species *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Pichia pastoris* have been used as hosts for the expression of GPCRs[10–22]. Expression in these cells confers several advantages, including ease of genetic manipulation of the host cell, inexpensive and convenient culturing conditions, short generation times, and few competing GPCR subtypes. As unicellular eukaryotic cells, they all possess secretory pathways that function in a similar fashion to those in mammalian cells. Like mammalian cells, both *S. cerevisiae* and *S. pombe* cells use GPCRs and G proteins to communicate with the external environment. The facility with which heterologous GPCRs make use of the secretory machinery and interact with the endogenous G proteins may, in part, determine the usefulness of each fungal species as host for the expression of GPCRs.

Many GPCRs expressed in *S. cerevisiae* are localised to the plasma membrane, where they form appropriate high-affinity agonist-binding sites indicative of functional coupling to heterotrimeric G proteins[10,18,19,21,22]. Certain other GPCRs appear to be inappropriately localised to intracellular compartments and may be degraded in the vacuole[13]. Targeting GPCRs to the plasma membrane via fusion with a cleavable leader sequence or to the amino terminus of the yeast α-factor receptor, Ste2, may prove to be useful in overcoming this difficulty[10,13]. In many cases, high-affinity agonist-binding sites, which require productive interaction between GPCRs and G proteins, were either not detected or were distinctly different and of lower affinity than those observed in native tissues[11–13,15]. The GPCRs expressed in *S. cerevisiae* cells bearing the endogenous heterotrimeric G protein also failed to produce detectable pheromone-induced signal transduction, probably due to inadequate receptor–G-protein interaction and low assay sensitivity[11–13]. Coexpression of GPCRs with cognate heterologous G_α protein or chimeric yeast–heterologous G proteins overcomes this difficulty for some GPCRs[10,18,21].

The expression of GPCRs in *S. pombe* has permitted high-affinity antagonist-binding sites to be detected[13,14,16]. High-affinity agonist binding to the human β_2-adrenergic receptor was observed in the presence of the native G_α protein, although measurements of downstream signal transduction were not reported[14]. In an effort to produce agonist-induced signal transduction, the human neurokinin NK2 receptor was coexpressed with heterologous $G_{\alpha q}$, $_\beta$ and $_\gamma$ subunits[16]. High-affinity NK-agonist binding was detected but signal transduction was not observed, perhaps owing to the production of inactive G_α subunits by the yeast cells.

P. pastoris cells have often been used to produce large amounts of protein for structural studies because of the relative ease with which high levels of expression can be achieved. Expression of the 5-HT_{5A} and μ-opioid receptors led to the detection of antagonist-binding sites in membrane fractions[17,20]. The expected rank-order-of-potency was observed with various agonists in competition binding analyses, although the agonist-binding sites obtained from saturation-binding analyses were of low affinity, consistent with a failure to interact productively with endogenous G proteins[17,20].

sites were detected at moderate levels in crude membrane fractions, indicating that the expression of heterologous GPCRs in fungal cells permitted assembly into a functional ligand-binding configuration. However, for many GPCRs, agonist binding sites could not be detected or exhibited low affinity, indicating that the expressed GPCRs failed to interact productively with the endogenous G protein. An important advance was made by King *et al.*, who reported activation of the pheromone-response pathway via coexpression of the human β-adrenergic receptor with $G_{\alpha s}$ in *S. cerevisiae* cells lacking Gpa1[10]. The agonist-bound receptor signaled through $G_{\alpha s}$, which in turn activated the downstream signal-transduction pathway, with consequent induction of pheromone-responsive reporter gene expression, apparent cell-cycle arrest and formation of mating-specific cell structures[10].

The cell-cycle-arrest phenotype elicited upon agonist activation makes genetic selections difficult to implement and renders this system unsuitable for HTS applications. In an improved yeast-expression system that is to be suitable for genetic analysis and useful in HTS applications, the cells must exhibit a conveniently observable phenotype that is dependent on agonist activation. Recently, yeast cells containing genetic modifications that permit agonist induction of yeast-cell growth have been constructed (Fig. 1b)[18,19,21,22]. Because yeast genetic selections usually require growth phenotypes in order to be useful, the terminal response of haploid yeast cells to mating pheromone, cell-cycle arrest, was eliminated by deletion of the *FAR1* gene. *FAR1* encodes a negative regulator of G1 cyclins and is thought to serve as the primary interface between the pheromone-response pathway and the cell-cycle regulatory machinery[23–25], and so its deletion should allow the induction of pheromone-responsive genes with continued cell growth[23]. Released from cell-cycle control, agonist-stimulated *far1* cells are free to grow in response to agonist.

Detecting the yeast cells that respond to agonist requires a second modification – the addition of a pheromone-responsive reporter gene. In order to confer a selectable growth phenotype, the gene encoding His3 (an enzyme required for histidine biosynthesis) was placed under the control of the pheromone-induced *FUS1* promoter by integration at the *FUS1* chromosomal locus[26]. Agonist activation of a heterologous GPCR in cells results in induction of the *FUS1–HIS3* reporter gene. In *far1* host cells, rendered otherwise incapable of synthesising histidine due to a defective *his3* gene, agonist activation of GPCRs induces His3 expression, allowing growth on medium lacking histidine.

The sensitivity of the yeast-cell-growth response to the presence of agonist may be enhanced by interrupting the desensitisation or adaptation processes induced in response to chronic stimulation of the pheromone-response pathway. The primary adaptation mechanism is mediated by the product of the *SST2* gene[27]. Sst2 (see Glossary) is the first known member of the RGS (regulator of G-protein signaling) family of GTPase-activating proteins that has been shown to play an important role in the desensitisation of a variety of mammalian GPCR signaling pathways[27]. Yeast cells lacking functional Sst2 exhibit hypersensitivity to the presence of pheromone and the inability to recover from pheromone-induced cell-cycle arrest. A distinct desensitisation function, independent of Sst2, is initiated by the pheromone receptors. Unoccupied pheromone receptors act, by a poorly understood mechanism, to reduce agonist-induced signal transduction[28,29]. Acting in concert, these mechanisms promote recovery from cell-cycle arrest and reentry into the cell cycle by reducing the signal transmitted through the pheromone-response pathway. To increase the sensitivity of the yeast-cell response to GPCR agonists, the *SST2* and *STE2* genes in *MATa* cells were deleted.

GPCRs are discriminating in their association with G proteins, choosing to interact productively with only selected subsets of G_α subunits. In order to accommodate GPCR–G-protein selectivity, the yeast G_α-protein gene, *GPA1*, was deleted and replaced with plasmid-encoded G_α-protein genes. This format provides flexibility in the expression system by allowing various forms of heterologous G_α proteins to be expressed. Wild-type and chimeric yeast-mammalian G_α proteins have been shown to be functional when expressed in yeast, forming productive interactions with heterologous GPCRs[10,18,21] and with the yeast βγ complex[8].

Agar-based bioassay of heterologous GPCRs expressed in yeast

Incorporation of the genetic modifications described above into selected haploid cells permitted the development of a sensitive yeast bioassay system for the discovery of compounds that act on GPCRs[18,19,21,22]. Initially, the $G_{\alpha i2}$-coupled rat somatostatin SST_2 receptor was tested for its ability to be functionally expressed and coupled to the yeast pheromone-response pathway[18]. Radioligand-binding analyses of the SST_2 receptor in membrane preparations from yeast cells containing a Gpa1–$G_{\alpha i2}$ chimeric G protein revealed a high-affinity somatostatin binding site (with ^{125}I-labeled somatostatin S-14), comparable to those observed in native tissues[18]. When the

Glossary

GHRH	growth-hormone-releasing hormone
GPCR	G-protein-coupled receptor
HTS	high-throughput-screening
MAP kinase	mitogen-associated-protein kinase
RGS protein	Regulator of G-protein signaling protein
SST_2 receptor	somatostatin receptor subtype 2
SST2/sst2	wild-type and mutant forms of the gene encoding the yeast **RGS protein**
Sst2	protein encoded by the ***SST2*** gene
Ste2	α-factor receptor in yeast
Ste3	**a**-factor receptor in yeast

SST_2-receptor-containing yeast cells were embedded in selective agar medium and assayed for the stimulation of the pheromone-response pathway, somatostatin induced a dose-dependent growth response (Fig. 2). Somatostatin applied to the surface of the agar plate diffused radially and bound to the expressed SST_2 receptor, resulting in its activation and consequent induction of *FUS1–HIS3* expression. The responding cells formed a dense growth zone that was readily detectable over the limited background growth of the *his3*⁻ cells produced by basal *FUS1–HIS3* expression. Somatostatin agonists stimulated growth with a rank-order-of-potency identical to their inhibitory effect on mammalian adenylyl cyclase[18]. Remarkably, the SST_2 receptor stimulated growth in cells containing only Gpa1, indicating that certain heterologous GPCRs may interact productively with the yeast G_α protein.

The sensitivity of the bioassay was improved by deletion of the *SST2* and *STE2* genes[18]. Although little growth-promoting signal was produced by cells containing Sst2 and Ste2, growth proceeded to a much greater extent in their absence, allowing picomole amounts of S-14 to be detected (Fig. 2). The growth-promoting effects of the two mutations appeared to be additive – elimination of both Ste2 and Sst2 gave rise to more growth than deletion of *SST2* alone. The effect of deleting Ste2 may be due to the abolition of the desensitisation pathway induced by unoccupied Ste2 or to removal of competition for G protein.

The yeast bioassay was employed to screen cyclic octapeptide somatostatin analogs prepared in the course of a structure–activity relationship (SAR) program[30]. The program was designed to identify modulators of serum growth-hormone (GH) levels from amongst novel somatostatin-receptor subtype-selective agonists and antagonists. Somatostatin is a major regulatory neuropeptide that regulates the synthesis and secretion of growth hormone from the pituitary gland through inhibition of adenylyl cyclase activity[31]. Somatostatin agonists reduce serum GH levels by binding to pituitary somatostatin receptors, including the SST_2 subtype. Somatostatin antagonists were predicted to increase serum GH levels by interfering with the activity of somatostatin. When assayed in yeast cells containing the rat somatostatin SST_2 receptor, agonist analogs elicited the expected growth-inducing response (Fig. 3). The analogs exhibited affinities for the SST_2 receptor comparable to S-14 and subtype selectivity, producing a greater-than-1500-fold preference for the SST_2 over the SST_3 receptor[30].

Somatostatin analogs with antagonist activity were tested using a modification of the agonist screening assay[30], in which somatostatin was added to the molten agar along with the yeast cells. This stimulated a uniform growth response in all cells in the plate. Antagonists pipetted onto the surface of the plate caused zones of growth inhibition around the application site (Fig. 3). Substitution of the L-cysteine residue at position 2 (analogs 1 and 3) with D-cysteine (analogs 2 and 4) converted these compounds from agonist to antagonists, while retaining receptor-subtype selectivity[30].

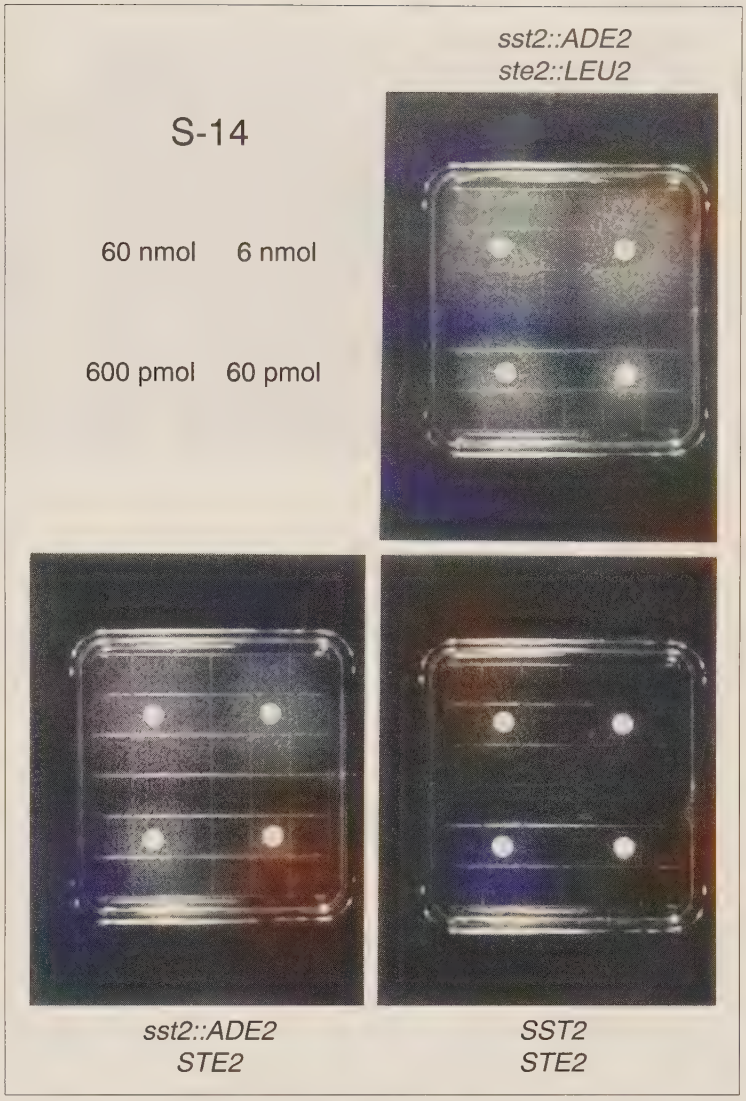

Figure 2

Yeast-cell growth in response to somatostatin agonists. Yeast cells containing the rat somatostatin SST_2 receptor and a Gpa1–$G_{\alpha i2}$ chimeric G_α protein [strains LY288 (*SST2 STE2*)[18], LY268 (*sst2 STE2*)[18] and LY364 (*sst2 ste2*; M. Pausch and L. Price, unpublished)] were stimulated to grow in response to application of somatostatin S-14. Comparison of the growth zones elicited by treatment with somatostatin indicates that the sensitivity of the yeast-growth assay can be substantially improved by deleting Sst2 and Ste2. Samples of the yeast-cell cultures described above were plated in molten, histidine-deficient agar medium[18]. Somatostatin S-14 was applied to the surface of the solidified agar in the stated amounts on sterile filter disks, and the plates incubated at 30°C for 48 h. Reproduced with permission from Ref. 18.

The properties of the somatostatin analogs were confirmed in radioligand-binding and cyclic-AMP-accumulation assays. These results indicate that the yeast screening assay can distinguish compounds that differ only in the stereospecificity of a single amino acid residue.

That the agar-based antagonist assay is useful in HTS applications was demonstrated by the isolation of a somatostatin antagonist from a synthetic combinatorial peptide library[32]. D amino acid peptide hexamers (6.4×10^7 unique peptides) divided among 400 separate pools were tested in the antagonist format. Those pools that displayed somatostatin-receptor-selective

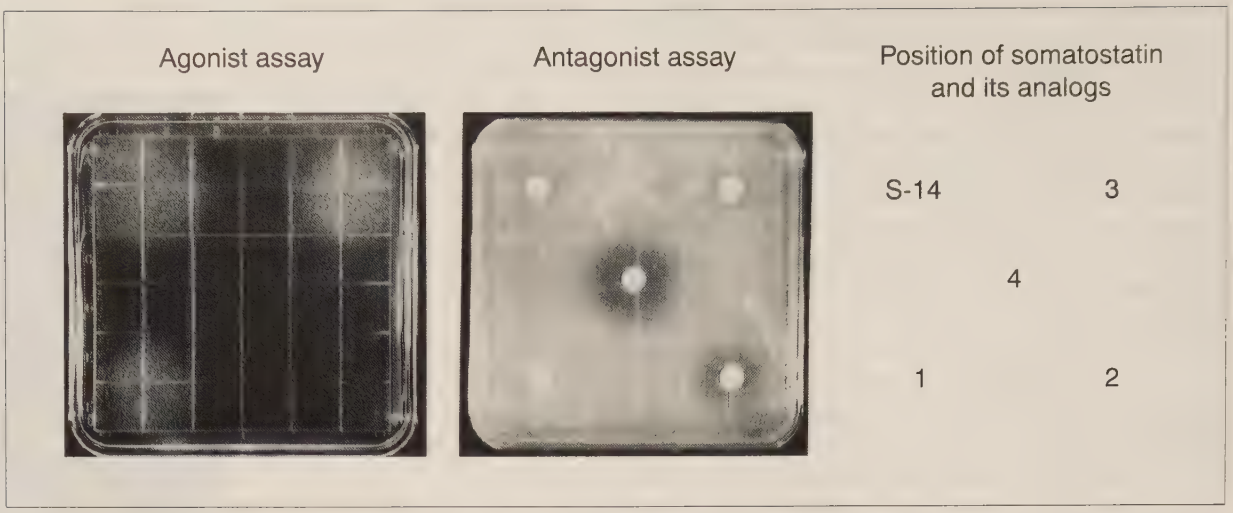

Figure 3

Yeast bioassay of somatostatin analogs possessing agonist or antagonist properties. Yeast cells that express heterologous G-protein-coupled receptors can be adapted to screen for either agonists or antagonists by simple modifications of the assay format. LY268 (*sst2 STE2*)[18] cells containing the rat somatostatin SST_2 receptor and a Gpa1–$G_{\alpha i2}$ chimeric G_α protein were plated in molten, histidine-deficient agar medium[18,30]. Agonist assay: samples (5 nmol) of somatostatin S-14 or its analogs (1–4)[30] were applied directly to the surface of the solidified agar in the positions shown. Antagonist assay: S-14 was added to the molten agar to a final concentration of 100 nM prior to plating; S-14 and its analogs (5 nmol samples) were applied to the surface of the solidified agar on sterile filter disks. The plates were incubated at 30°C for 48 h.

Somatostatin S-14: Ala–Gly–c[Cys^2–Lys–Asn–Phe–Phe–Trp–Lys–Thr–Phe–Thr–Ser–Cys]–OH

Analogs:
1 H_2N–4-NO_2–Phe–c[Cys^2–Tyr–D-Trp–Lys–Val–Cys]–Tyr–NH_2
2 H_2N–4-NO_2–Phe–c[D-Cys^2–Tyr–D-Trp–Lys–Val–Cys]–Tyr–NH_2
3 AcNH–4-NO_2–Phe–c[Cys^2–Tyr–D-Trp–Lys–Thr–Cys]–Tyr–NH_2
4 AcNH–4-NO_2–Phe–c[D-Cys^2–Tyr–D-Trp–Lys–Thr–Cys]–Tyr–NH_2

The superscript numbering refers to the location of the D/L-cysteine residue at position 2 in each of the analogs. Reproduced with permission from Ref. 30.

growth-inhibitory properties were deconvoluted by repeated antagonist assays in four iterative steps. The sequence of the resulting pure D amino acid hexapeptide is distinct from somatostatin or any of its analogs, yet possesses potent somatostatin-antagonist properties, confirmed in radioligand-binding and adenylyl-cyclase-inhibition assays[32]. In a rat model of regulated GH levels, the somatostatin antagonist administered alone induced GH release, reversed the effects of somatostatin agonist when coadministered, and potentiated growth-hormone-releasing-hormone (GHRH or growth-hormone-release factor, GRF) activity[32]. Thus, the yeast screening technology is capable of identifying dry lead compounds with desired biological effects from large compound libraries.

GPCR bioassays in liquid growth media

Agar-based bioassays provide convenience and high throughput, and, in particular, give a qualitative assessment of the potency and efficacy of test compounds. However, quantitative measurements of receptor activation of the pheromone-response signal-transduction pathway are often performed in assays of β-galactosidase expressed from reporter constructs composed of LacZ sequences fused to a pheromone-responsive promoter, such as *FUS1–LacZ*. These procedures often require cell lysis or permeabilisation steps that are cumbersome to implement in a HTS format. A simple, convenient alternative involves measuring the increase in optical density of agonist-induced yeast cell cultures in liquid medium[19], which has the additional advantage of being compatible with computerised data-handling systems.

Agonist compounds that activate the G_s-coupled rat adenosine A_{2a} receptor were assayed in cultures grown in 96-well microtiter dishes (Fig. 4a)[19]. Yeast cell growth, which proceeds in a logarithmic fashion, may be measured using a conventional microtiter dish reader fitted with a 620 nm filter and translated into dose-response curves (Fig. 4b). Agonists with varying affinities for the rat adenosine A_{2a} receptor activated a maximal growth rate at concentrations equivalent to their potency in activating their cognate effector enzyme, adenylyl cyclase. This type of assay could detect agonists possessing affinities that vary over a 100-fold range, such as the adenosine-receptor agonists NECA (K_i = 35 nM) and CHA (K_i = 3400 nM), while distinguishing compounds with as little as a twofold difference in affinity[19].

The yeast cells used in liquid assay of the rat adenosine A_{2a} receptor contained Gpa1 as the G_α-protein component[19]. Surprisingly, a large number of heterologous GPCRs interact with the yeast heterotrimeric G protein sufficiently strongly to induce a growth-promoting signal, including the rat G_i-coupled somatostatin SST_2 receptor[18], the rat G_s-coupled

adenosine A_{2a} receptor[19], the human growth-hormone-releasing-hormone receptor[21] and the rat G_q-coupled cholecystokinin CCK_B receptor[22]. Certain G_α proteins, for example, Gpa1 and $G_{\alpha15/16}$, can couple to several different types of GPCR[33]. Thus, the simplest test of heterologous GPCRs expressed in yeast may be conducted by examining their ability to couple to the endogenous G_α protein. When agonist-dependent signalling is not observed, Gpa1 may be replaced with a cognate heterologous G_α protein or a chimeric yeast–mammalian G_α protein.

Two-hybrid assay of GPCR–ligand interactions

Certain classes of GPCR possess ligand-binding determinants in a large N-terminal domain, for example the secretin and GHRH receptors. The N-terminal domain of the GHRH receptor has been expressed in the yeast two-hybrid system and its ability to mediate an interaction with its cognate hormone evaluated[21]. A fragment encompassing the complete N-terminus of the human GHRH receptor was fused to one half of the two-hybrid Gal4 protein and GHRH itself was fused to the other half. When expressed in appropriate yeast cells, the GHRH receptor domain and GHRH formed a protein–protein interaction sufficient to promote a growth response in the two-hybrid system[21]. The specificity of the interaction was demonstrated by the introduction of mutations into the GHRH domain that are known to interfere in the interaction with GHRH; these mutations also abolished the two-hybrid interaction[21]. This approach may be extended to other members of the secretin class of GPCRs and provides a potentially useful alternative method for investigating receptor–ligand interactions.

Future prospects

The state of the art in pharmaceutical drug discovery requires mechanism-based screening assays of high selectivity and throughput. Screening assays implemented using yeast strains functionally expressing heterologous GPCRs are very useful in this regard. The diversity of GPCRs successfully expressed in yeast to date indicates that the technology will have broad applicability. Yeast cells functionally expressing the rat somatostatin SST_2 receptor have been used successfully to screen somatostatin analogs and combinatorial libraries to identify novel subtype-selective agonists and antagonists with potent effects *in vivo*[30,32]. These screening approaches can be implemented immediately for the increasingly large number of heterologous GPCRs that couple directly to the yeast G_α protein. Expanded analyses will be facilitated by coexpression of cognate mammalian G_α proteins. Modified G_α proteins that improve functional coupling to the GPCR and $\beta\gamma$ subunit complex may be predicted based on the recently determined G-protein crystal structures[34,35]. Assays employing a growth phenotype may also be used to identify agonists that interact with orphan GPCRs. In this system, orphan GPCRs are expressed in yeast and a panel of selected agonists, compounds from chemical files and/or combinatorial libraries are applied in an effort to identify a surrogate ligand. As a potentially useful alternative, yeast expression libraries designed for the secretion of random small peptides have been designed. When the secreted peptide derived from the library plasmid is expressed in yeast along with a GPCR, an autocrine loop is established, resulting in the growth of cells that express an active peptide[36]. Using this scheme, novel agonists and antagonists of the yeast α-mating-type pheromone receptor were identified, suggesting it may also be useful for heterologous GPCRs[3,36].

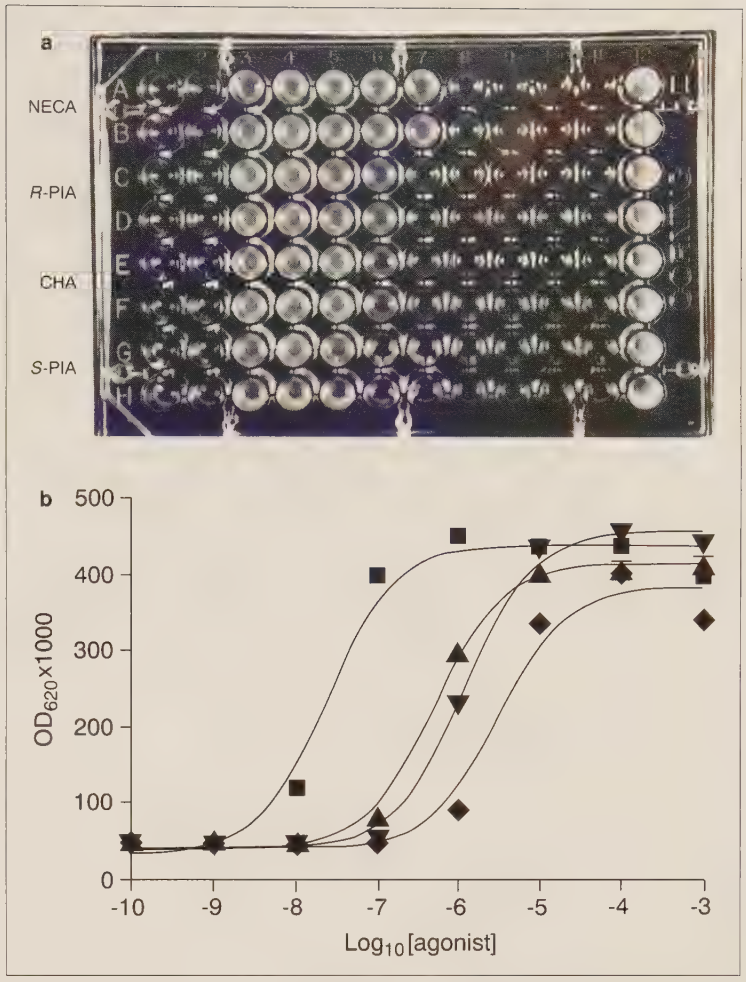

Figure 4

Agonist-stimulated growth of yeast measured in liquid bioassays. Growth assays conducted in small-scale liquid cultures have the advantages of quantitative measurement of agonist growth-promoting properties and facile data-handling capability. (**a**) LY594 cells expressing the rat A_{2a}-adenosine receptor were cultured in 200 μl histidine-deficient medium in the wells of a 96-well microtiter dish[19]. Samples of the indicated adenosine agonists were added to duplicate wells to give final concentrations in the range 10^{-10} M to 10^{-3} M. The plates were agitated at 30°C for 16 hours. Growth was measured by determining the optical density of cultures at 620 nm. L-Histidine (60 mg l^{-1}) was added as a positive growth control. (**b**) The dose-response curve was plotted using paired values obtained during the logarithmic phase of growth. The rank-order-of-potency of the compounds is identical to values obtained in assays of agonist-induced adenylyl-cyclase stimulation. Symbols: ■, NECA; ▲, *R*-PIA; ▼, CHA; ◆, *S*-PIA. Abbreviations: NECA, 5-N-ethylcarboxamidoadeno-sine; *R*-PIA, *R*(–)-N6-(2-phenylisopropyl)adenosine; *S*-PIA, *S*(+)-N6-(2-phenylisopropyl)adenosine; CHA, N6-cyclohexyladenosine; [agonist] represents the molar concentration of the agonist. Reproduced with permission from Ref. 19.

An additional advantage of a heterologous-GPCR-expression system in yeast is the availability of yeast genetic approaches to examine structure–function issues. Genetic selections based on the induced growth phenotype may be used to identify mutations that illuminate aspects of GPCR function, such as improvement of heterologous GPCR interaction with the yeast G protein, induction of constitutive GPCR activity and the response to noncognate agonists. A genetic approach was used to identify constitutively active mutants of the yeast α-factor receptor, and this could be employed with heterologous GPCRs[37]. Similar approaches should be useful for the dissection of the interactions within the heterotrimeric G-protein complex, with RGS proteins, and with downstream effector enzymes. Yeast cells contain functional versions of adenylyl cyclase, phospholipase C, a high-affinity potassium-uptake transporter and a potassium channel. In principle, conditional phenotypes could be complemented by corresponding mammalian isotypes, leading to the construction of entirely synthetic signal-transduction pathways in yeast cells.

Acknowledgments

I thank the present and former members of the Molecular Genetic Screen Design and Molecular Biology Groups of American Cyanamid Company for stimulating discussions, John Hadcock for critical reading of the manuscript, and Don Kirsch for his continuing support.

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focus

Electrochemical regeneration of redox enzymes for continuous use in preparative processes

Wim A. C. Somers, Wim van Hartingsveldt, Edwin C. A. Stigter and Jan Pieter van der Lugt

The use of oxidoreductases for preparative purposes must be accompanied by an efficient process for the regeneration of the enzyme following the reaction process. Electrodes have been exploited as a source of this oxidative or reductive energy for a large number of redox enzymes. Although direct interaction between electrodes and enzymes is rare, oxidoreductases can interact readily with electrode surfaces via an appropriate intermediate (or mediator). Various methods for the preparative application of redox enzymes using electrochemical regeneration have been proposed; these vary in their capacity to meet the demands posed on a preparative process.

Redox enzymes catalyse a variety of oxidation and reduction reactions that are of interest for preparative synthesis. Several methods have been developed for the regeneration of these enzymes, which is an important prerequisite for their application; these are mostly based around either the biochemical or the electrochemical regeneration of the enzyme. The first of these, in which the cofactor or coenzyme is regenerated by a second enzyme has been previously reviewed[1,2] and will not be considered here. Electrochemical regeneration, commonly referred to as bioelectrochemistry, can be defined as 'the technology in which redox enzymes are regenerated by an electrochemical device', that is, the enzyme is coupled to an electrode as the ultimate source of oxidative or reductive power (see Glossary).

The application of redox enzymes is flourishing, particularly in the development of amperometric biosensors, in which the enzymes are regenerated by a wide variety of electron acceptors. Over 80 substrate–redox-enzyme systems have been described[3], and the field is rapidly expanding. Whereas in sensor development, the linearity of reaction, sensitivity and stability of the systems are the predominant parameters, in preparative applications, high volumetric productivities and the stability of the reaction systems are of greater importance[4].

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Bioelectrochemical regeneration of oxidoreductases

During an oxidation reaction catalysed by an oxidoreductase, electron equivalents are transferred between the substrate and the enzyme. Regeneration of the enzyme is then performed using an appropriate electron acceptor; in bioelectrochemistry, this is achieved by mediated transport of the electron equivalents (Box 1). The mechanisms by which enzymes interact with mediating compounds is still a poorly studied topic. Therefore the building of bioelectrochemical reactors and the choice of the mediator is still an empirical field[5], to which more attention should be paid. A number of mediators, however, interact with large groups of dye-linked dehydrogenases, generally with fast kinetics. Ferrodicyclopentadienyls (ferrocenes), osmiumbipyridyls and various viologens (bipyridinium salts) are among the most frequently used mediators. These compounds interact readily with large numbers of flavoproteins, quinoproteins and various NAD(P)-dependent enzymes[5,6].

Electro-enzymic reactors

The application of electrochemical devices to the preparative synthesis of biomolecules has been rare. The design of enzyme reactors deals with such factors as the efficiency of enzyme use, the lifetime of the enzyme and the recovery of the reaction products. In addition, the volumetric productivity of the systems has to be economically feasible. A variety of reaction principles are exploited, but the basis of electrochemical regeneration remains the same for all systems: an electrode is the source or sink of electron equivalents.

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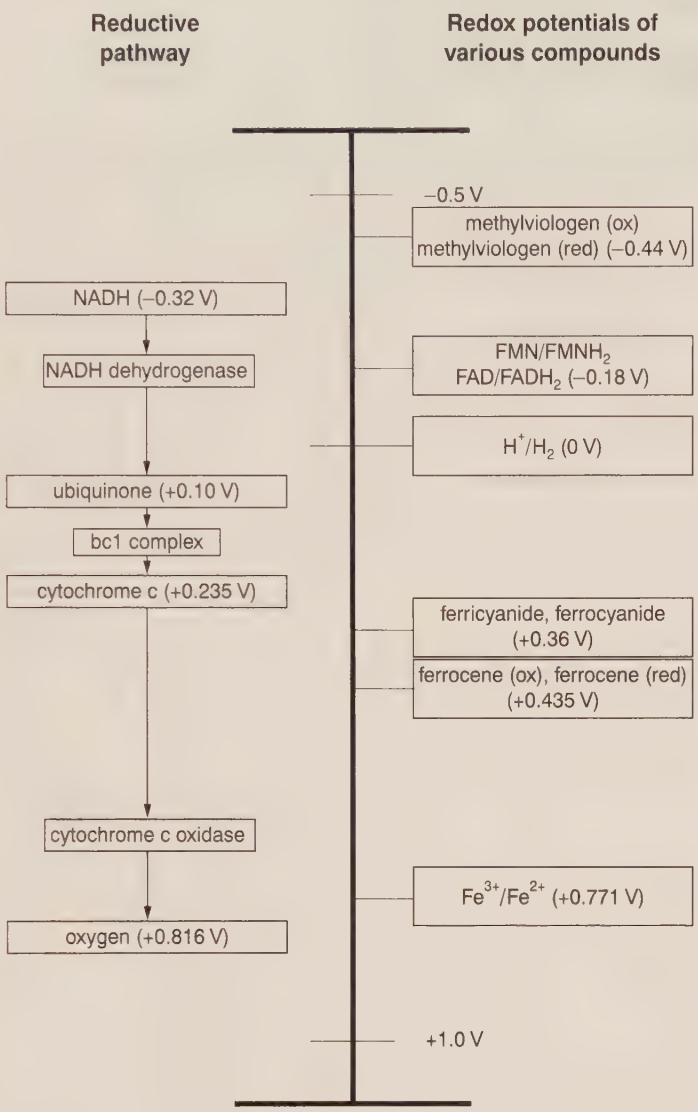
PII: S0167-7799(97)01119-0

Box 1. The aerobic respiratory chain and the electron transport between redox enzymes and electrodes.



Oxidation–reduction reactions are characterized by the transfer of electrons from an electron donor (the reducing agent or reductor) to an electron acceptor (the oxidizing agent or oxidator). In general, effective regeneration of a redox enzyme can therefore be achieved by introducing a species to the reaction system with enough reductive or oxidative power to oxidize or reduce the reduced or oxidized enzyme. If one considers the following equilibrium:

$$\underset{\text{(oxidizing agent)}}{\text{electron acceptor}} + e^- \leftrightarrow \underset{\text{(reducing agent)}}{\text{electron donor}}$$

the redox potential for the reaction:

$$2\,H^+ + 2\,e^- \leftrightarrow H_2$$

is by convention set to 0 V, at defined conditions of concentration and pH. A lower redox potential therefore represents a stronger reducing couple. For example, dithionite and methylviologen will reduce NAD^+ to NADH. This general principle is then used for the development of regeneration processes for oxidoreductases (see figure above). An electrode system that is poised at an appropriate potential will allow the transport of electrons from the enzyme to the electrode via the mediator. Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NADH, nicotinamide adenine dinucleotide.

The interaction between enzyme and electrodes is then achieved by a redox-active component that shuttles the electrons between the enzyme and the electrode (Box 1).

Several approaches are employed to achieve bioelectrochemical catalysis (Fig. 1). The simplest involves bringing together the enzyme and an electroactive species that acts as a mediator in an electrochemical cell[7]. The bioelectrochemical conversion then can be conveniently monitored by the electric current, the current being related to the amount of product formed by the following equation:

$$\text{moles product} = \frac{I \times t}{F \times n}$$

in which I = current (A), t = time (sec), F = Faraday's number (96 494 Coulomb mol^{-1}), n = number of electrons transferred per mole of product.

This principle allows the selectivity and specificity of the bioelectrochemical reactions to be monitored, as employed in a number of studies[7–9]. Table 1 gives an overview of studies dealing with systems that have a potential for preparative applications and the productivity that characterizes them.

A two-chamber bioelectrochemical reactor (Fig. 2) was used for the continuous regeneration of coenzyme (NADH) in the production of gluconate from glucose with a glucose dehydrogenase[10] and acetaldehyde from ethanol with an NADH-dependent alcohol dehydrogenase[11]. Reaction equilibria can be shifted to the product side by regenerating NADH. Complete oxidation of the substrate was not directly achievable owing to product inhibition in the batch-operated systems. The maximum product yields observed for this approach were 0.04 mol m^{-3} h^{-1}. An improvement in the productivity of this type of reactor is achieved by the immobilization of the enzyme. Immobilization processes increase the ease of product recovery from

Glossary

Bioelectrochemistry	An enzymatic reaction, especially of an **oxidoreductase**, in which electrons are transferred to or from an electrode surface.
Bioelectrocatalysis	A mechanism that increases the rate of half-cell reactions at the electrode surface, involving a biochemical or biotechnological catalyst.
Electrocatalysis	A mechanism that increases the rate of half-cell reactions at the electrode surface.
Mediator	A redox couple that shuttles electrons from the redox centre of an enzyme to an electrode, or vice versa.
Oxidoreductase	An enzyme catalysing an oxidation and/or reduction reaction.

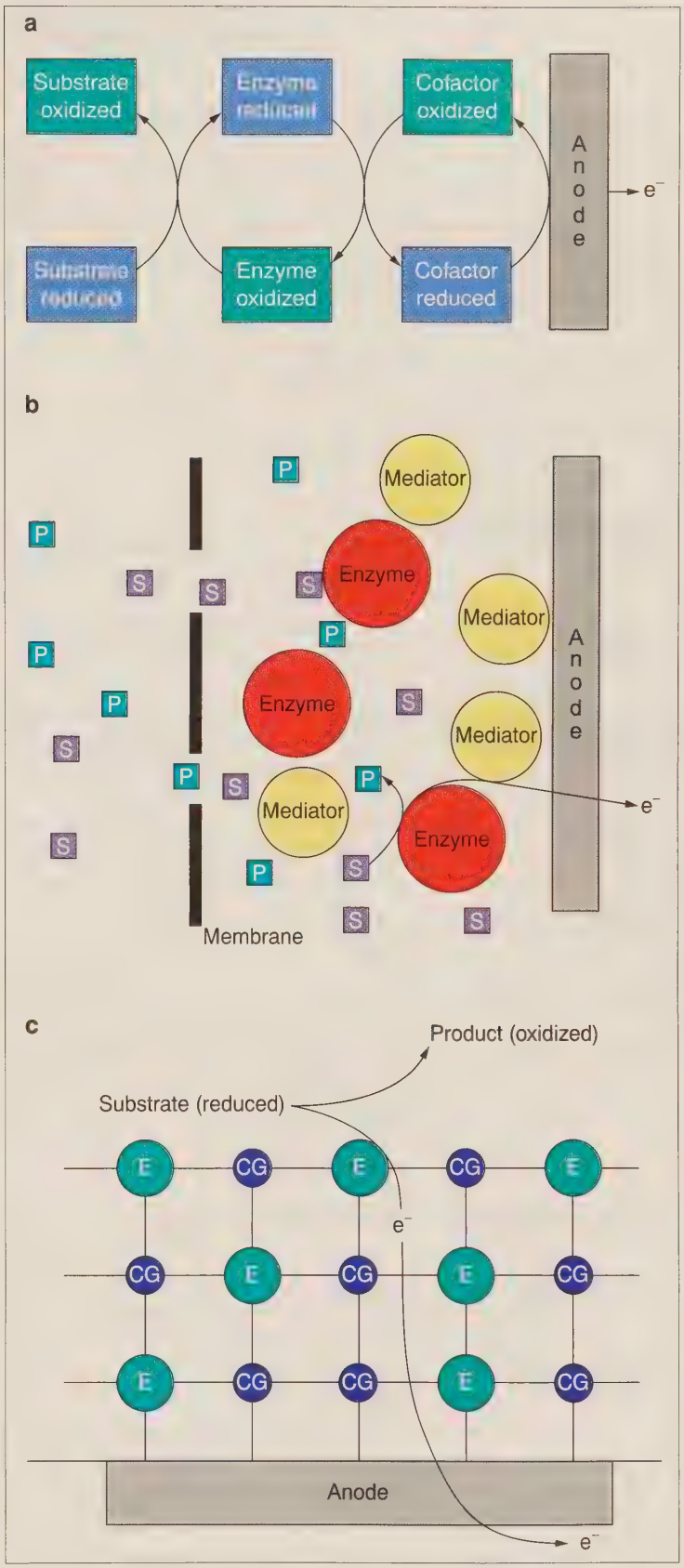


Figure 1
Reactor configurations for bioelectrocatalysis. Several methods have been developed to establish a bioelectrochemical process and assure a continuous process. (**a**) Soluble mediated processes – both the enzyme and the mediator are in soluble form. The mediator shuttles the electrons between the redox center of the enzyme and the electrode. Substrates and products are present in the process fluid, necessitating the development of an appropriate downstream process. (**b**) Immobilized membrane process – both the enzyme and the mediator are in soluble form. The mediator is present in a high molecular weight configuration. Membranes allow the separation of biocatalyst and mediator from the substrate and product. S represents substrate, P represents product. (**c**) Immobilized enzyme electrode – both the enzyme and the mediator are (co)immobilized on the electrode surface, allowing both effective conversion in terms of mass transport and effective separation of the biocatalyst and mediator from the substrate and product (on-line downstream processing). E represents enzyme, CG represents the conducting group.

the reaction mixture. In addition, a higher concentration of the active form of the coenzyme (or mediator) in the vicinity of the enzyme can be achieved in immobilized enzyme electrodes. This is an advantage in terms of reaction kinetics and results in improved reactor performance. Complete immobilization of the enzyme and mediator is also advantageous, because it avoids contamination of the product with the mediator.

Partially immobilized systems, in which either the enzyme or the mediator are immobilized, are the first examples of this approach. For example, the conversion of glucose into gluconic acid in a two-chamber bioelectrochemical reactor has been demonstrated[12]. In this device the biocatalyst (glucose oxidase) was immobilized as a monolayer on a carbon-felt electrode with a high surface-area:volume ratio (35 000 $m^2 m^{-3}$). The maximum current densities were of the order 0.5 $A m^{-2}$ using the redox couple benzoquinone–hydroquinone as a soluble mediator regenerated at the enzyme electrode. The reactor produces approximately 100 g gluconate from glucose at a working potential of 0.35 V versus a standard Calomel electrode (SCE). The enzyme was extensively stabilized (by a factor of 10) by the use of the artificial electron acceptor benzoquinone, which replaces oxygen in the reaction cycle[13] and consequently prevents the production of peroxide.

Membrane reactors, in which the enzyme and the mediator are immobilized by entrapment in a membrane module, lack the kinetic advantages mentioned above, but have apparent advantages in terms of on-line product recovery. This principle was shown for the conversion of *p*-hydroxybenzylalcohol into *p*-hydroxybenzaldehyde using a poly(ethylene glycol)-based ferrocene mediator with a molecular weight of approximately 20 000. The system operated satisfactorily without loss of activity for four days, but no conclusive data were presented regarding the productivity of the system[14]. An advanced immobilized system in which two enzymes and the mediator were all immobilized has recently been described[15] in which batchwise oxidation of benzyl alcohol into benzaldehyde was achieved at an electrode consisting of ferrocene, diaphorase and an NADH-dependent alcohol dehydrogenase immobilized on a polyacrylic-acid layer. While the dehydrogenase is oxidizing the alcohol to the aldehyde, the diaphorase is

Table 1. Preparative electro-enzymic devices

Conversion	Enzyme	Mediator	Productivity		Remarks	Refs
			Current density ($A\,m^{-2}$)	(Moles product $l^{-1}\,hr^{-1}$)		
Soluble systems						
β-D-glucose → D-glucono-lactone	glucose dehydrogenase	NAD^+/NADH	0.03	0.036	NAD^+ regeneration	26
ethanol → acetaldehyde	yeast alcohol dehydrogenase	NAD^+/NADH	0.04	0.044		11
L-lactate → D-lactate	L-lactate dehydrogenase	NAD^+/NADH	0.03	0.033	Process includes electrochemical reduction of pyruvate into D,L-lactate	27
(Partially) immobilized systems						
glucose → gluconate	glucose oxidase (i)	benzoquinone (s)	0.5	0.55		12
alcohols → aldehydes/ketones	diaphorase (i) + alcohol dehydrogenase (i)	ferrocene (i) + NAD^+/NADH (s)	nd	2.5		15
glucose-6-phosphate → 6-phosphogluconate	glucose-6-phosphate dehydrogenase (i)	phenazine methosulfate (i)	2.55	0.0007		28
4-ethylphenol → *p*-cresol	*p*-cresolmethyl-hydroxylase (i)	α,-bis-methyl-ferrocene PEG (i)	nd	nd	Membrane reactor used for immobilization	14
enoates → acylates	enoate reductase (i)	methyl viologen (s)	1.0	nd	Immobilization of enzyme in calcium alginate	7
$NAD(P)^+$ → NAD(P)H	VAPOR (i)	viologens (i)	0.002	nd		23
nitrate → nitrogen	nitrate reductase, nitrite reductase, nitrogen-oxide reductase, dinitrogen-oxide reductase (i)	not identified (i)	nd	0.4	Denitrification of drinking water with four enzymes	25
hydrogen production	hydrogenase (i)	methyl viologen (i)	0.1	nd		24
glucose → gluconate	glucose oxidase (i)	osmium bipyridyl (i)	3.3	nd		29
alcohol → aldehyde, ketone	QH-ethanol dehydrogen-ase (i)	osmium bipyridyl (i)	0.4	nd	Enantioselective conversion	20
lactose → lactobionate / glucose → gluconate	PQQ-glucose dehydrogenase (i)	osmium bipyridyl (i)	30	nd		21,22

(i) immobilized; (s) soluble; PQQ, pyrroloquinoline quinone; QH, hydroquinone; VAPOR, viologen-accepting pyronucleotide oxidoreductase; nd, not determined.

regenerating NAD^+ with oxidized ferrocene as the electron acceptor. The coenzyme is soluble in this system, which thus still neccesitates the separation of the product from the coenzyme. The productivity is however relatively high, of the order 2.5 (moles product) $l^{-1}h^{-1}$.

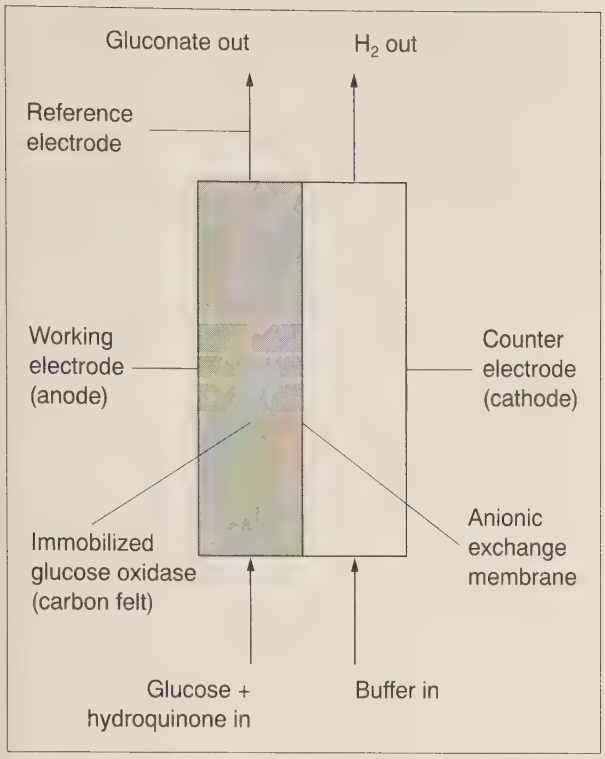


Figure 2
Bioelectrochemical reactor with immobilized glucose oxidase for the production of gluconate (adapted from Ref. 12).

Immobilized enzyme electrodes for preparative use

Immobilized enzyme electrodes containing the mediator in the vicinity of the enzyme are attractive for kinetic and process-development reasons. In addition, to use enzyme electrodes for preparations, a high current density is one of the most important requirements. To obtain nonleaking enzyme electrodes with a high electron-transfer rate, the enzyme and mediator can be integrated in a number of ways, for example modification of the enzyme with redox mediators, modification of the electrode surface with a mediator, coupling the enzyme to this modified electrode (Fig. 1), and covalent binding of the coenzyme itself to an electrode surface and reconstitution of the apoenzyme[16]. The use of redox polymers (in which mediators such as ferrocene derivates, viologens or osmium complexes are attached to polymer backbones such as polysiloxane, polypyrrole or polyvinylpyridine[17]) is highly attractive. The complex of polyvinylpyridine with osmium bis(bipyridine) allows very efficient transfer of electrons from a large variety of oxidoreductases with varying cofactors [such as pyrrolo-quinoline quinone (PQQ), FAD, porphyrines and metals] to the electrodes[18–20]. These electrodes are characterized by appreciable and, for preparative purposes, feasible current densities (Table 1). The electrode incorporating the redox polymer and a PQQ-dependent glucose dehydrogenase[21,22] showed the highest current density ever reported for an enzyme electrode or a bioelectrochemical device (28 A m^{-2}). The conversion of glucose and lactobionate with this electrode was demonstrated on a small scale[22], the productivity of this system being 60 times higher than in previously described bioelectrochemical systems (see Table 1).

For reduction reactions, polymers with a relatively low redox potential have been developed (Box 1). One example is the direct electron transfer between a viologen-accepting pyronucleotide oxidoreductase (VAPOR) coimmobilized with a number of viologens in the continuous reduction of $NAD(P)^+$. A productivity of 3.6×10^{-5} (moles of NADH) $m^{-2}h^{-1}$ (equivalent to a current density of 2×10^{-3} A m^{-2}) was observed, but considered to be in the lower range of productivities for industrial purposes[23]. A comparable redox polymer with a viologen functionality has also been used for the mediation of a hydrogenase enzyme[24]. One of the most innovative applications of this technique is the bioelectrochemical denitrification of drinking water. In an immobilized reactor incorporating nitrate reductase, nitrite reductase, nitric-oxide reductase and dinitrogen-oxide reductase, complete conversion of nitrate into nitrogen gas was achieved on millilitre scale with a conversion ratio of 25 (kg nitrate) $m^{-3}h^{-1}$ (that is, 400 moles $m^{-3}h^{-1}$) and a reactor half-life of three months[25]. This application and configuration clearly shows that bioelectrocatalysis has considerable potential in numerous applications. With such volumetric productivity, the system is clearly superior to the recently developed microbial conversions. In addition, as nitrogen is the ultimate reaction product, the technology is preferable to physicochemical techniques, in which large amounts of waste chemicals are produced[30].

Conclusions

The development of processes for the continuous use of oxidoreductases is progressing rapidly. In the development of efficient reactor setups, it is important to obtain a better understanding of electron transfer within proteins. Further research is needed to obtain data on the mechanism of interaction between cofactors and/or mediators with enzymes. In addition, more knowledge about long-range electron transfer within and between proteins will aid the development of efficient electron-transfer systems, which will, in turn, improve the current densities of enzyme electrodes. A better understanding of these fundamental questions may also help the rational use of synthetic redox polymers, stimulate the development of systems in which 'wired' coenzymes or suitable mediators are used to functionalize enzymes that lack an efficient electron-transport system and the speed of development of enzyme electrodes in which the oxidoreductases can be exploited to their full catalytic activity.

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Bacterial biosensors for monitoring toxic metals

Sridhar Ramanathan, Mark Ensor and Sylvia Daunert

Biosensors utilize biological components to provide selectivity for monitoring compounds of environmental, clinical and industrial importance. A number of biosensors based on bacteria have recently been developed for monitoring toxic metals in the environment. The advantages and disadvantages of these types of biosensors are discussed.

Many of the analytical techniques presently used for monitoring pollutants require expensive equipment and extensive pretreatment of environmental samples[1]. Recognition of the difficulties inherent in classical analytical techniques has spurred the development of novel sensing systems that use a biological component as the sensory element. These biosensors offer great sensitivity and selectivity for the detection and quantification not only of compounds of environmental importance but also of clinical and industrial importance. Potential advantages of biosensor technology include economy, portability and the possibility of identifying and quantifying specific compounds directly in complex mixtures found in the air, soil or water and in biological samples. In many cases, these complex mixtures would require pretreatment before they could be assayed by classical analytical methods.

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Biosensors using intact cells

A biosensor combines a biological component (the sensing element), which is responsible for the selectivity of the device, with a detection system (the transducer) for measuring the reaction of the biological component with the substance (analyte) being monitored. The biological component can be an enzyme, antibody, receptor or even whole cells; the biological reaction detected can be the activity of an enzyme, the binding of an analyte to an antibody or receptor, the induction of gene expression within cells or even cell death. Detection systems for measuring these biological events usually rely on electrochemical[2] or optical[3–11] principles.

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PII: S0167-7799(97)01120-7

There are a number of advantages to using intact microorganisms, rather than isolated biological components, in biosensors[12,13]. Microorganisms are usually more tolerant of assay conditions that would be detrimental to an isolated enzyme or protein: because microorganisms have mechanisms that enable them regulate their internal environment, they are more tolerant of suboptimal pH and temperatures than purified enzymes, and are also less likely to be inhibited by solutes in the sample being assayed. Microorganisms are cheaper to use because the active biological component does not have to be isolated and, because microorganisms are living, unlimited quantities can be prepared relatively inexpensively.

An analyte, such as a pollutant, must be taken up by the microorganism before a response is produced, and so using intact microorganisms can provide information about the bioavailability of the analyte. Classical analytical methods of detection cannot distinguish between bioavailable and unavailable pollutants; because many toxic compounds accumulate in living organisms, it is important to determine the bioavailability of these compounds present in the environment[3,5,10].

In addition to the typical disadvantages encountered using isolated biological components in sensors, which are primarily problems of stability and lifetime of the biological component, the use of intact microorganisms produces a few other concerns: for example, biosensors using intact cells usually have longer response times than biosensors that use isolated biological components. They may also need a longer time to return to the base-line signal after use, and so reversibility may be a problem[12,13]. There is also the possibility of variation in the responses of biosensors produced from different batches of cells. Therefore, growth conditions must be monitored closely to ensure that each batch of cells is as similar as possible. Culture age, temperature, cell density and aeration of the culture during the induction of the response can all affect the response of the biosensor. Despite these problems, which are not insurmountable, the advantages offered by using intact cells has led to the development of a number of biosensors of this type.

This review will focus on recent progress that has been made in the development of biosensors relying on intact bacterial cells to monitor toxic metals. Both nonspecific and specific biosensors that utilize intact cells have been developed for this purpose.

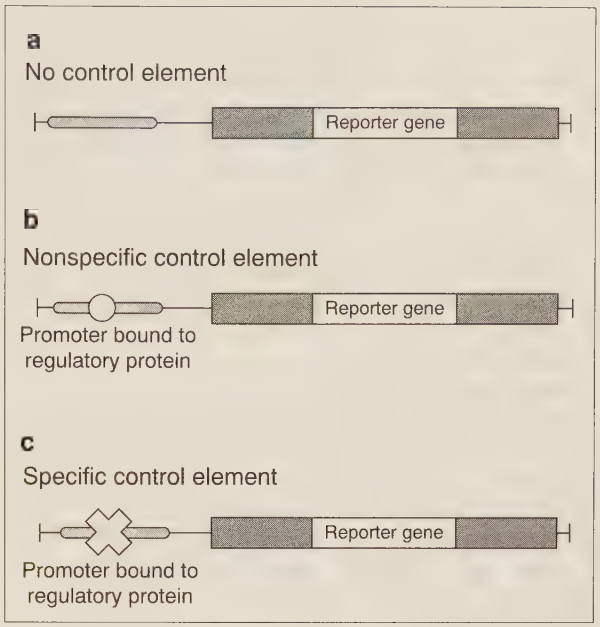

Figure 1

Sensing systems based on bacteria. (**a**) Expression of the reporter gene is regulated by a constitutive promoter resulting in continuous production of the reporter protein by the cells. When the cells are exposed to toxic substances, there is a corresponding reduction in signal due to cell death or inhibition of cell metabolism, and thus of reporter-protein production. This type of sensing system is not specific for detecting a particular type or class of toxin, but is useful for general screening of samples for any compound that is toxic to the cells. (**b**) In this type of sensing system, the reporter gene is expressed under the control of a promoter (e.g. heat-shock or stress promoter) that is responsive to a wide range of conditions that are potentially harmful to the cells. Exposure of the cells to a wide variety of toxic substances will result in expression of the reporter gene and a positive signal. (**c**) The reporter gene is under the control of a promoter that will respond only to very specific toxic compounds. The promoter can be positively or negatively regulated by a regulatory protein. In positive regulation, when the compound being measured binds to the regulatory protein, it promotes the binding of the regulatory protein to the promoter and induces transcription of the reporter gene. In negative regulation, the regulatory protein is bound to the promoter in the absence of the toxic compound, inhibiting transcription. The regulatory protein is released from the promoter when it binds the toxic compound allowing transcription of the reporter gene to occur.

Nonspecific biosensors

Toxin-sensitive cells that express a reporter gene constitutively can be used as a biosensor for the nonspecific detection of toxins (Fig. 1a). When the cells are exposed to critical amounts of toxins, either the cells' metabolic activity decreases or the cells die. In either case, as the concentration of toxins in the cells' environment increases there is a corresponding decrease in the signal produced by the reporter gene. It should be stressed that this type of biosensor will respond to any type of substance that is toxic to the cells. The biosensor cannot be used to determine the type of toxin present.

Based on this principle, simple biosensors have been developed for determining the biotoxicity of a sample. The commercially available Microtox® is based on the inhibition of the bioluminescent response of the bacterium *Vibrio fischeri* when exposed to toxic substances[14–16], including toxic metals. When a number of metals were tested with the Microtox® reagent, the order of increasing toxicity was found to be $Cr^{6+}<Ni^{2+}<Cd^{2+}<Zn^{2+}<Pb^{2+}<Cu^{2+}$ (Ref. 17). Increasing the exposure time of the bacteria to the metal enhanced the sensitivity of the assay.

Nonspecific biosensors have also been developed that are based on the heat-shock or stress response

displayed by many organisms[18,19]. Organisms have a number of general and specific mechanisms involving alterations in gene expression that they use to respond to unfavourable growth conditions. Exposure to heat, toxic compounds and toxic metals can induce the expression of stress-response genes. The induction of reporter genes linked to stress promoters that respond to a wide variety of toxic compounds can be used to produce biosensors useful as first indicators of the presence of pollutants in the environment (Fig. 1b).

Specific biosensors

Bacterial resistance to metals

Microorganisms have evolved a variety of mechanisms that allow them to survive and grow in environments containing levels of metals that would be toxic to most organisms. Bacterial strains have been isolated that show a high level of resistance to metals such as zinc, copper, nickel, cadmium, mercury, chromium, lead and cobalt[20–23].

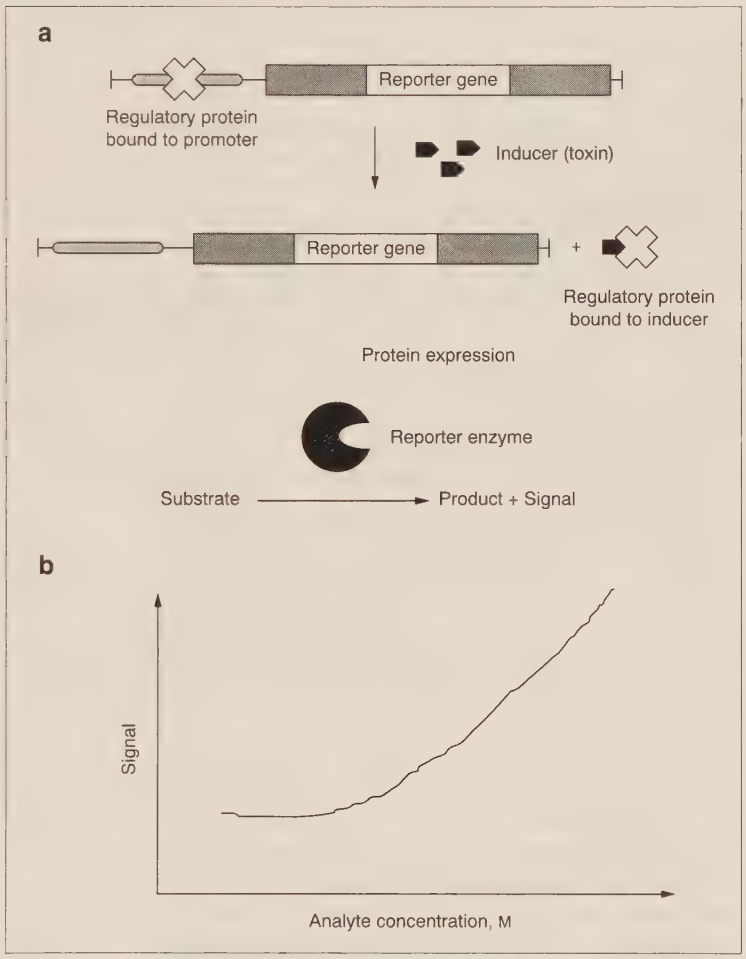

Figure 2

The sensory process in a sensing system based on bacteria. In this example, (**a**) the regulatory protein binds to the promoter in the absence of a specific inducer (toxin), preventing expression of the reporter gene. When the inducer is present, it binds to the regulatory protein, releasing the reporter gene from repression by the regulatory protein. This results in the transcription of the reporter gene, production of the enzyme and hence of a signal. (**b**) The intensity of the signal obtained by the activity of the reporter enzyme can be used to determine the concentration of the analyte (toxin) present once the relationship between them has been calibrated.

There are at least five different mechanisms that can allow a microorganism to become resistant to metals[24,25]: (1) exclusion of the metal from the cell by the cell membrane; (2) active transport of the metal from the cell so that toxic levels of the metal cannot accumulate inside the cell; (3) sequestration of the metal inside the cell so that it is not available to produce its toxic effects; (4) extracellular sequestration, in which the metal is bound to the outside of the cell, preventing it from entering the cell; and (5) chemical modification of the metal to a form that is less toxic to the cell. Within a single bacterium, one or more of the above mechanisms may be responsible for resistance to a given metal.

The genes responsible for these resistance mechanisms are organized in operons, usually found on plasmids carried by the resistant bacteria. In many cases, these plasmids will confer resistance to more than one toxic metal. Usually, the expression of the resistance genes is tightly regulated and induced by the presence of specific metals in the cell's environment. Because of the specificity of this regulation, the promoters and regulatory genes from these resistance operons can be used to construct promoter–reporter-gene fusions for use in the development of biosensors that can detect toxic metals.

The general approach for producing a biosensor using intact cells is to fuse an inducible promoter from one of the metal-resistance operons to a reporter gene (Figs 1c and 2); reporter genes include those that code for bioluminescent proteins, such as luciferase, and for β-galactosidase, which can be detected electrochemically using an electrode or by using chemiluminescent substrates (Fig. 3). The light produced by bioluminescent proteins can be measured by a variety of instruments, including photometers, luminometers, charged-couple devices and liquid-scintillation counters. Light emitting bacteria can be used in conjunction with fibre-optic cables that can transmit the light to a detection device. A more detailed description of how bioluminescence can be measured and some of the shortcomings involved can be found in a review by Burlage and Kuo[26].

A number of biosensors using this promoter–reporter-gene concept have been developed for the detection of metals. The promoter regions of operons that are responsive to metals such as mercury[3–6], copper[10], aluminium[9–11], nickel[10], cadmium[7], antimonite and arsenite[2,7,8] have been fused to the genes for luciferase or β-galactosidase (Fig. 3). Biosensors for the detection of mercury[3–5] and one capable of detecting both antimonite and arsenite[2] will be described in more detail as examples of how cells can be engineered for the detection of specific analytes.

Biosensors for the detection of mercury

Resistance to mercury can be found in both Gram-negative and Gram-positive bacteria. Resistance in these bacteria is encoded by genes of the *mer* operon[28] (Fig. 4a), which code for proteins that are involved in the transport of mercury [Hg(II)] into the cell and for

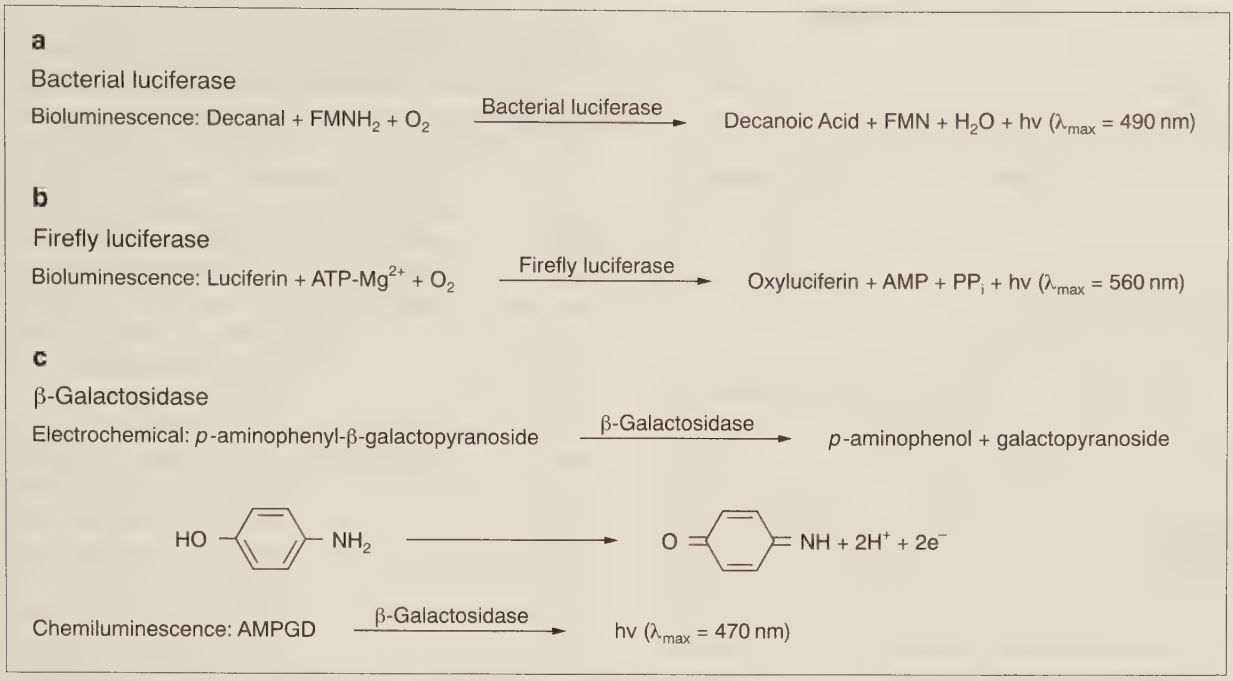

Figure 3

Reactions catalysed by the three major reporter enzymes that have been used in sensing systems based on bacteria. (**a**) The light-emitting reaction of bacterial luciferase involves the oxidation of reduced riboflavin phosphate ($FMNH_2$) and a long-chain aldehyde (for example, decanal), resulting in the production of blue–green light. (**b**) Firefly luciferase produces light by oxidizing luciferin and requires ATP. (**c**) β-Galactosidase activity can be detected either by electrochemical methods or by chemiluminescence. Electrochemical detection involves lysing the bacterial cells and adding the substrate, *p*-aminophenol-β-D-galactopyranoside, to the cell lysate; the concentration of the product of the reaction, *p*-aminophenol, is then determined electrochemically with an electrode. β-Galactosidase activity can also be measured by using a chemiluminescent substrate such as 3-{4-methoxyspiro[1,2-dioxetane-3,2′-tricyclo-{3.3.1.1^{3,7}}decan]-4-yl}phenyl-β-D-galactopyranoside (AMPGD)[37].

the transformation of the toxic form of mercury, Hg(II), to the less-toxic elemental form, Hg(0); Hg(0) is volatile and evaporates out of the cell.

Regulation of the *mer* operon is controlled by two proteins, MerR and MerD, encoded by the *mer*R and *mer*D genes, respectively[29]. MerR regulates operon expression in three ways: (1) in the absence of mercury, MerR binds to the promoter region of the *mer* operon and prevents transcription of the *mer* genes; (2) when Hg(II) is present, MerR activates initiation of transcription of the *mer* operon; and (3) MerR represses its own transcription, regardless of whether Hg(II) is present[29]. MerD can also bind to the promoter region of the *mer* operon, but with a much lower affinity than MerR; while MerR controls the basal level of expression of the *mer* operon, MerD acts as a repressor to limit the maximum level of *mer* expression. The regulatory components of the *mer* operon from *Escherichia coli* have been used in the construction of several biosensors for the detection of mercury[3–5].

The importance of understanding how metal-resistance mechanisms work and how the genes coding for them are regulated is demonstrated in the construction of three recombinant strains with different detection limits for Hg(II). Selifonova *et al.*[3] constructed three separate plasmids containing different portions of the *mer* operon fused to the *lux*CDABE bacterial luciferase genes. These plasmids were used to transform *E. coli* and produce three recombinant strains with varying detection limits for Hg(II). Induction times of 40 min and 100 min were examined for each of the three strains. The selectivity for Hg(II) of the three bacterial strains was not reported because other metals were not tested.

The first plasmid, pRB28, contains only the *mer* promoter and *mer*R gene fused to the *lux* operon. Cells containing this plasmid were induced significantly when exposed to Hg(II) concentrations as low as 1 nM, and maximum induction was achieved with 0.5–1.0 μM Hg(II).

The second plasmid, pOS14, contains the *mer*T and *mer*C genes in addition to the promoter and *mer*R gene. The *mer*T and *mer*C genes code for proteins that transport Hg(II) into the cell. However, cells containing this plasmid are not resistant to mercury because they still lack the enzymes for transformation of Hg(II) to the less-toxic Hg(0) form. The detection limit of these cells for Hg(II) was 0.5 nM and the cells were responsive between 0.5 nM and 0.5 μM; the transport of Hg(II) into the cytoplasm makes the cells sensitive to lower concentrations of Hg(II) in the environment than cells containing pRB28. This may be due to the fact that, at a given external concentration of Hg(II), MerR is exposed to higher concentrations of Hg(II) in pOS14-containing cells than in cells lacking the Hg(II) transport proteins, thereby inducing greater luciferase expression.

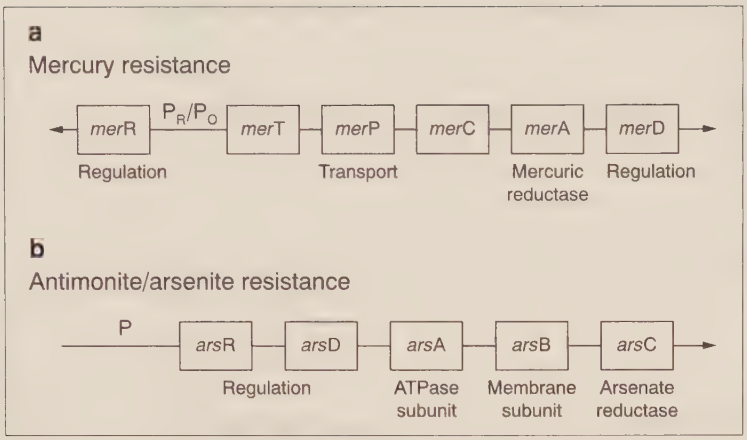

Figure 4

Operons for resistance to (**a**) mercury and (**b**) antimonite and arsenite. (**a**) The *mer* operon is regulated by the *mer*R and *mer*D gene products, MerR and MerD, respectively. The expression of *mer*T, *mer*P, *mer*A, *mer*C and *mer*D is controlled by the same *mer* promoter (P_O), resulting in a polycistronic message. The transcription of the *mer*R gene is regulated by a second promoter (P_R) and occurs in the opposite direction of that producing the polycistronic *mer* message. In the absence of mercury, MerR binds to the *mer* promoter preventing transcription of the mer genes. In the presence of Hg(II), MerR remains bound to the promoter but there appears to be a shift in the MerR–promoter complex that allows transcription to occur. MerD can also bind to the promoter region of the *mer* operon, but with a much lower affinity than MerR. MerD acts as a repressor to limit the maximum level of *mer* expression. (**b**) The *ars*D and *ars*R genes code for proteins, ArsD and ArsR, respectively, that regulate the expression of the *ars* operon. In the absence of antimonite and arsenite, ArsR is bound to the *ars* promoter (P). Arsenite and antimonite induce expression of the *ars* operon by binding to ArsR and promoting its release from the promoter. Unlike *mer*R, *ars*R is expressed from the same promoter that controls expression of the other genes in the *ars* operon, and is part of a polycistronic *ars* message. ArsD controls the upper limit of *ars* expression by inhibiting transcription of the operon.

The third plasmid, pOS15, contains the entire *mer* operon. Cells with this plasmid can transform Hg(II) to Hg(0) and are resistant to Hg(II) at higher concentrations than cells containing either of the first two plasmids. Because of this resistance, these cells can be induced at concentrations of Hg(II) that are toxic to the first two strains, but their response is not as sensitive. These cells produce a significant signal to Hg(II) at concentrations ranging between 25 nM and 1 μM.

E. coli containing pRB28 and pOS14 were used to examine bioavailable mercury in contaminated water flowing in and out of a lake[3]. Mercury levels determined by cells containing either plasmid were measured at approximately 20 nM for water flowing into the lake and 1 to 2 nM for the outlet water. Cold-vapour atomic-fluorescence spectroscopy measured 20 nM (inlet), similar to that measured with the cells, and 7 nM (outlet) of mercury, indicating that the proportion of bioavailable mercury in the total mercury pool decreased during retention of the water in the pond.

Tescione and Belfort[4] have developed a minireactor and minisensor using recombinant bioluminescent cells responsive to Hg(II). The minireactor consisted of a water-jacketed (for temperature regulation) reaction chamber that holds a total volume of about 100 ml. A glass tube with an optical fibre was inserted into the chamber so that light production could be monitored. The minisensor had a total capacity of 3 ml. These two biosensors utilized *E. coli* cells in which the *lux* operon had been placed under the regulation of the *mer* promoter and MerR. In the minireactor, after a 30 min induction with mercury, light emission increased in a linear fashion with Hg(II) concentrations ranging between 20 nM and 4 μM. Salts of zinc, iron, copper, cobalt and aluminium failed to induce light production, thus demonstrating the selectivity of the minireactor for mercury. Using the minisensor design, the lower limit of detection was improved to 10 nM and the response was still linear up to 4 μM.

As demonstrated in the above examples, the detection limits and response range of a biosensor can be improved by selecting appropriate regions of the metal-regulated operon for construction of the biosensor and also by the physical design of the biosensor itself. A third way of improving a biosensor is by selecting a reporter gene that produces a stronger signal. This was demonstrated by the improved detection limits for mercury in a system that uses firefly luciferase instead of bacterial luciferase[5]. The *mer*R gene and *mer* promoter were fused to the gene for firefly luciferase and an assay for mercury developed using a microtitreplate format. Using this system the limit of detection was 0.1 fM Hg(II). The luminescence increased with increasing Hg(II) concentration in a linear manner up to 0.1 μM, after which the luminescence decreased rapidly to background levels owing to the toxic effect of Hg(II) on the cells. Cd(II) also induced luminescence, but the concentration needed for induction was almost 10^7 times greater than that of Hg(II). Zinc, copper, manganese and cobalt failed to induce luminescence even at millimolar concentrations.

The detection limit obtained with this system is about a million-fold lower than those reported by Selifonova *et al.*[3] and Tescione and Belfort[4]. Possible explanations for this increased sensitivity include the difference in the quantum yields of firefly and bacterial luciferase (about 90% for firefly luciferase as opposed to about 5% for the bacterial enzyme) and the higher turnover number of firefly luciferase.

A biosensing system for antimonite and arsenite

When exposed to antimonite or arsenite, resistant bacteria express a protein pump that can transport these metals out of the cell, preventing their buildup to toxic levels[29]. The genes encoding this protein pump are located on the chromosome of *E. coli*[30], as well as on plasmids found in certain strains of *E. coli*[31] and *Staphylococcus*[32].

The *ars* operon of pR773, present in certain strains of *E. coli*, consists of five genes: *ars*A, *ars*B, *ars*C, *ars*D and *ars*R[33,34] (Fig. 4b). The *ars*A and *ars*B genes code for the proteins that form the pump, which is capable of transporting both antimonite and arsenite out of the cell; arsenate cannot be transported by this pump, but *ars*C codes for an enzyme, arsenate reductase, that reduces arsenate to arsenite, which can then be removed by the pump. The *ars*R and *ars*D genes code

for proteins that regulate the expression of the *ars* operon. In the absence of antimonite and arsenite, the regulatory protein, ArsR, is bound to the *ars* promoter. Arsenite and antimonite induce expression of the *ars* operon by binding to ArsR and causing its release from the promoter, thus allowing transcription to occur[35,36]. ArsD is an inducer-independent *trans*-acting factor that controls the upper limit of *ars* expression by inhibiting transcription of the operon.

The regulatory components of the *ars* operon have been used to construct a plasmid (pBGD23) in which the *ars* promoter, *ars*R, and a segment of the *ars*D gene are fused to a β-galactosidase reporter gene[2]. In cells containing pBGD23, expression of the β-galactosidase gene is regulated by the ArsR protein and is responsive to both arsenite and antimonite. The production of β-galactosidase is monitored with a substrate that can be detected electrochemically using an electrode (Fig. 3c). The sensitivity of the *ars*–β-galactosidase system was found to increase with increasing times of induction: the limit of detection was 10 μM for antimonite using a 30 min induction, improving to 0.1 μM when the induction was carried out for 17 h. This increase in sensitivity may occur because, at longer induction times, very low concentrations of antimonite induce the formation of sufficient amounts of β-galactosidase to produce a detectable signal. Similar results were obtained using arsenite. The response of the system to bismuth, carbonate, nitrate, phosphate and sulfate was at least 100-fold lower than to antimonite.

Concluding remarks

There is a demand for simple and sensitive analytical techniques that can monitor the levels of metals in environmental samples directly. The development of biosensors that can detect specific metals at low concentrations may help to meet this demand. Information on the presence and bioavailability of metals is critical for monitoring the bioremediation of toxic waste sites.

In order to make useful biosensors for monitoring metals, it is of vital importance to understand the mechanisms that cells employ to regulate metal-responsive operons and, hence, resistance to metals; this, and the transport and metabolism of metals within cells, are factors that determine the intracellular concentration of metals, which, in turn, affects the sensitivity and selectivity of biosensors that utilize responsive cells. A greater understanding of these resistance mechanisms may make it possible to customize biosensors for a given environment or application, perhaps by selecting different regulatory systems or by creating mutations that alter the sensitivity and selectivity of the microorganisms used in the biosensor.

The biosensors for mercury and arsenite/antimonite described here detect their respective analytes in concentration ranges that are useful for environmental monitoring. They are also specific for their target analytes as potentially competing cations do not cause interference even at relatively high concentrations.

There are several drawbacks to most microbial biosensors that have been developed so far that may limit their applications. First, the cells require relatively long induction times (usually longer than 30 min) to produce a measurable response; as a result, optimization usually requires a compromise between maximum sensitivity and quick response time. In some cases it will be possible to reduce the response time by using a reporter gene, such as that for firefly luciferase, whose product can be detected in extremely low amounts so that only a few minutes are required for the cells to make enough of the reporter enzyme to detect. Second, it normally takes a considerable time for the cells to return to the base-line signal so that the biosensor can be reused. This is a serious limitation in applications where a series of samples need to be rapidly assayed. Third, these biosensors have not been extensively tested using real environmental samples and the effects of microorganisms or inhibiting compounds in these samples that could interfere with signal production have not been determined. Only extensive testing of each individual biosensor system will determine its usefulness for measuring real samples.

Microorganisms represent a wide genetic diversity that can be exploited in the development of biosensors that offer great sensitivity and selectivity. Despite the drawbacks, biosensors have considerable potential in applications for the detection and quantification of a wide variety of compounds that would otherwise be difficult to monitor.

Acknowledgments

Work on the development of engineered bacteria for the detection of arsenite and antimonite was supported by grants to S. Daunert from the National Science Foundation (CHE-95022299) and the National Institutes of Health (NIEHS with funding provided by the Environmental Protection Agency (1P42ES07380).

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Ultrasonic separations in analytical biotechnology

W. Terence Coakley

Cells in megahertz-frequency noncavitating ultrasonic standing waves concentrate at submillimetre distances and are, as large clumps, easily removed from suspension in flow or batch systems. An ultrasonic filter for perfusion hybridoma culture is now available. Results from small-volume prototype analytical-scale systems can inform the design of effective filter or batch-clarification systems for a wide range of cell sizes, concentrations and sample volumes. Large increases in the rates of aqueous biphasic separations and of the rates and sensitivities of analytical immunocoated particle-agglutination assays occur in standing waves. Ultrasonic manipulation is briefly compared with immunomagnetic and dielectrophoretic separations.

Biotechnologists require the ability to manipulate cells in suspension, for purposes including cell harvesting, cell retention in perfusion fermenters and in-line clarification of fermenter samples for analytical purposes. While centrifugation and filtration play clear roles in meeting such requirements, these techniques also suffer from numerous limitations – centrifuges are not readily compatible with in-line systems, while solid filters can suffer from blockage. However, systems that exploit the forces acting on cells in ultrasonic standing waves can remove cells from suspension in a manner that can readily be employed in-line and is not limited by physical blockage[1,2]. The rates of aqueous biphasic cell separations can be markedly increased by attractive interdroplet forces in ultrasonic standing waves[3,4]. In further analytical applications, the forces on cells or coated microparticles in a standing wave can be exploited to improve the detection levels and rates of immunoagglutination tests for antigen or antibody[5].

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Forces on cells in ultrasonic standing waves

An ultrasonic standing wave is the sum of two oppositely directed travelling waves[6]. These waves may originate from two independent transducers or from a transducer and a reflector (Fig. 1). In a standing wave, the pressure amplitude has maximum (antinodal) and zero (nodal) values twice over a distance of one wavelength. A discontinuity in the propagating phase, for example a particle, cell, droplet or bubble, acquires a

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PII: S0167-7799(97)01122-0

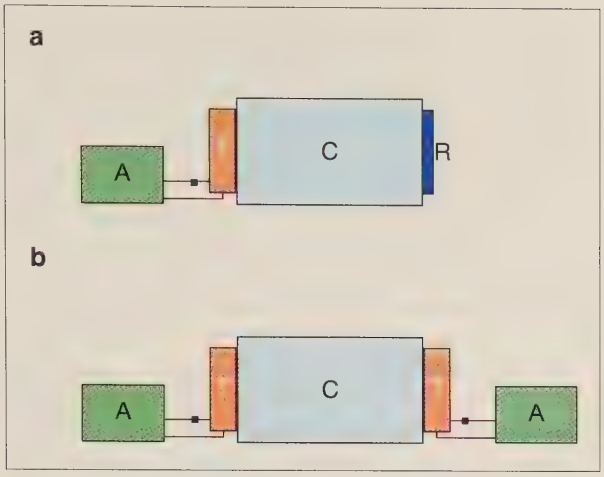

Figure 1
Schematic diagrams of arrangements to produce ultrasonic standing waves in aqueous suspensions. The piezoelectric ultrasonic transducer (T) is often a short ceramic of circular or rectangular cross section (i.e. a disc or a plate). Thin conducting layers on the circular or rectangular surfaces act as electrodes. The ultrasonic frequency whose wavelength is twice the thickness of the transducer is its thickness-resonance frequency. When a potential difference, from the amplifier A and alternating at this frequency, is applied to the transducer electrodes, the transducer continuously propagates a mechanical wave through the suspension in the container (C). A standing wave, with pressure-antinodal planes parallel to the transducer and separated by half a wavelength, is set up in the container when a travelling wave interacts with another wave travelling in the opposite direction from either (**a**) a reflector (R) or (**b**) a second transducer. The container is coupled to the transducer(s) and/or reflector either directly or, indirectly, through a liquid or solid phase.

position-dependent acoustic potential energy by virtue of being in the sound field[6,7]. Suspended particles therefore tend to move towards and concentrate at positions of minimum acoustic potential energy. For cells, these localized regions are generally close to pressure nodes[8], which are separated from each other by distances of half a wavelength (Fig. 2). For the case where the cell diameter is small compared to the ultrasound wavelength, the 'primary'[9,10] radiation force, F_r, acting on a cell of volume V_c located at a distance z from a pressure node is derived from the gradient of the particle acoustic potential energy[7], and is given by Eqn 1:

$$F_r = -[\pi P_o^2 V_c \beta_w/(2\lambda)] \cdot \phi(\beta,\rho) \cdot \sin(4\pi z/\lambda) \quad (1)$$

where P_o is the peak acoustic pressure amplitude and λ is the wavelength of sound in the aqueous suspending phase, which has a compressibility β_w. The function $\phi(\beta,\rho)$ equals $[(5\rho_c - 2\rho_w)/(2\rho_c + \rho_w) - (\beta_c/\beta_w)]$, where β_c is the compressibility of the cell and ρ_c and ρ_w are the densities of the cell and the suspending phase, respectively. Secondary forces[10] drive the concentrated cells to the local minima of the pressure amplitude, within the pressure nodal planes, to give regions of cell concentration that appear as columns of clumps striated at half-wavelength separations (Fig. 2). Although the principal features of cell concentration in a standing wave can be semiquantitatively explained by a combination of primary and secondary forces, further refinement and extension of this theory may be necessary to explain factors such as the high dependence of the concentration efficiency on biomass concentration[2,11].

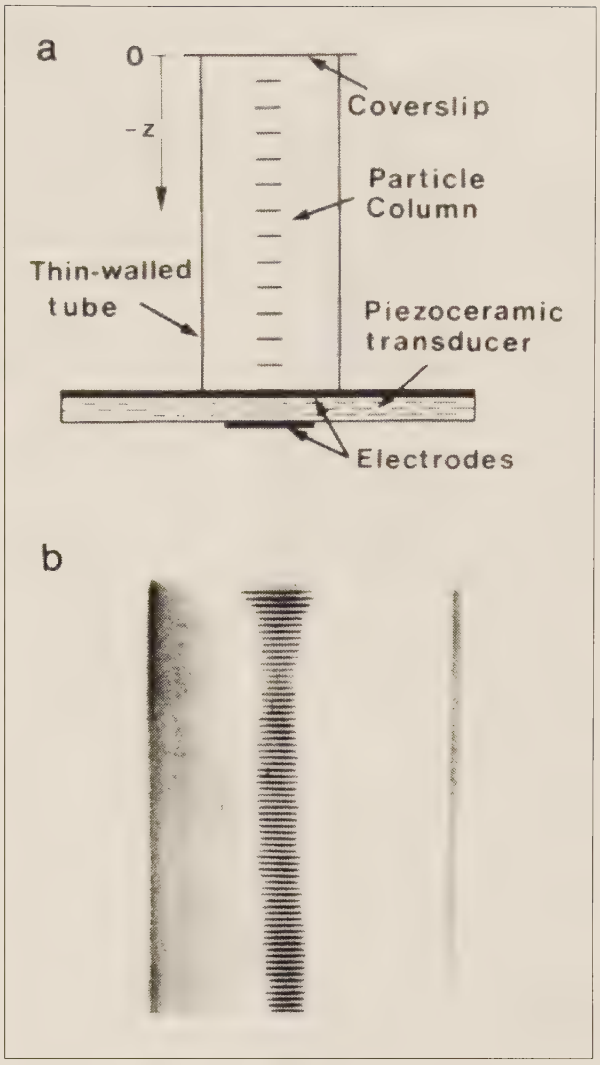

Figure 2
A particular example of the concentration of particle clumps at half-wavelength (0.25 mm) separations in a 3 MHz standing wave. (**a**) A 12 mm diameter cylindrical tube containing a uniform suspension of 9 μm diameter polystyrene beads was placed on a transducer whose back-electrode area was reduced so that the resulting standing wave had its half-wavelength-separated particle-potential-energy minima on the axis. (**b**) The particles moved with axial and radial velocity components to concentrate in axial clumps surrounded by clarified aqueous phase. Reprinted (abstracted) with permission from Whitworth, G. and Coakley, W. T. (1992) 'Particle column formation in a stationary ultrasonic field' *J. Acoust. Soc. Am.* 91, 79–85. Copyright 1992 Acoustical Society of America.

Experimental concentration of cells in a standing wave

The primary force (Eqn 1) is dependent on both the properties of the sound field (pressure amplitude, P_o, and wavelength, λ), which are established by the

experimenter, and the properties of the cell (volume, V_c, and the density and compressibility relative to those of the suspending medium). Ultrasonic frequencies of greater than 1 MHz (λ <1.5 mm) are preferred for cell manipulation, so that high pressure amplitudes can be employed without inducing ultrasonic cavitation, with its associated vigorous order-disrupting bubble activity. Most cells have densities in the range $1.05 - 1.15 \times 10^3$ kg m^{-3}. Weiser and Apfel[12] obtained a value of 0.76 for β_c/β_w for single erythrocytes, but the compressibility of single cells has not been widely measured. It can be shown that a cell with a density of 1100 kg m^{-3} in a 3 MHz standing wave of peak pressure amplitude 0.5 MPa will experience a maximum force 120 times that of gravity (Eqn 1). Biotechnologists' familiarity with centrifugal fields of this magnitude will suggest that, over quarter-wavelength distances (0.12 mm at 3 MHz), manipulation of eukaryotes should be readily achieved, although bacterial manipulation will be more difficult and molecular manipulation virtually impossible in a standing wave with the above specification. This ranking of ease of manipulation can also be appreciated by noting that the volume dependent force F_r (Eqn 1) that drives a cell is 1000 times greater for a eukaryote of radius 10 μm than for a prokaryote of radius 1.0 μm while the radius-dependent viscous drag that retards cell movement is only ten times greater for the eukaryote.

A further factor influencing ultrasonic manipulation of single cells is that suspending phases are rarely at rest, so that the cells within a container are subject to convection by thermal currents. Flow due to environmental temperature gradients increases from about 5 to 250 μm s^{-1} when container dimensions increase from millimetre to centimetre scales (M. A. Grundy, PhD thesis, University of Wales, Cardiff, UK, 1994). Ultrasonic energy input introduces 'acoustic streaming'[13] and can increase temperature-gradient induced streaming. The induction of order in a suspension (as in Fig. 2) requires that cells be held against such background streaming. Single cells of radius 10 or 1.0 μm can be held in a typical 3 MHz standing wave field of pressure amplitude 0.5 MPa against a background flow of 0.8 mm s^{-1} or 8 μm s^{-1}, respectively. This highlights the importance of high prokaryote cell concentrations to provide clumps, large particle-equivalent bodies that can be held in flow. Because of the above considerations, particular attention is paid to minimizing acoustic streaming[10] and temperature-gradient flows[2,14] in ultrasonic cell-separation systems through cooling and attention to the quality of the acoustic resonance[1].

Separation of concentrated cells from suspension

To date, the general approach to cell separation in standing waves has been to harness the acoustic forces to form clumps, which can be more easily manipulated. Cell and particle suspensions have subsequently been clarified in batch[11,15–17] or flow[1,2,10,14,18,19] systems by clump collection through electronic modulation of the sound field[11,15,17,19] or pulse-controlled sedimentation[15,16]. Flowing suspensions have also been clarified by gravitational sedimentation of cell clumps retained in an ultrasonic filter (USF) without electronic modulation[1,2,10,18]. In systems of the latter type, the transducer and reflector planes, as well as the resulting pressure-nodal planes, are vertical (Fig. 3). A cell suspension flows into the filter system below the transducer housing and the cells are trapped in the nodal planes as the suspension flows upwards between the transducer and reflector. Cell packing proceeds until the clumps become too heavy and sediment within the nodal planes, remaining essentially intact as they drop below the inlet level[1,2].

Hybridoma perfusion cultures have been clarified in a recently developed sedimentation USF[10] at flow rates of litres per hour. The device is placed in the outlet line at the top of a fermenter, so that cells clump and sediment back into the culture[10]. The open nature of the filter addresses the protein-adsorption[20] and fouling[21–23] problems associated with various forms of solid filters. The active volume of the above filter is 75 ml. A more compact (7.5 ml) sedimentation USF can recover yeast with an efficiency of over 99%[2,18], while its smaller volume makes it possible to achieve 82% recovery of *Escherichia coli*[2] at flow rates of up to 3 ml min^{-1}. Ultrasonic frequency and the flow-rate-dependent residence time of a cell in the filter determine the effectiveness of a sedimentation filter, particularly for bacteria[2].

Analytical applications require filters with a much smaller active volume, and this reduction can be achieved by, for instance, shortening the acoustic pathlength between the transducer and reflector. A reduction in pathlength leads, for a constant volume-flow rate, to (1) an increase in the velocity of the suspension through the chamber and (2) a reduction in the chamber-residence time available to concentrate cells at sound-pressure nodes. As a consequence of the first of these results, increased viscous drag on cell aggregates and clumps causes them to pass through the chamber, to emerge as ordered layers in the outflow channel. The essential difference between this type of separator and the sedimentation or field-modulated batch or filter systems is that, here, the particles emerge with the outflow but in an ordered state. Work continues on the development of downstream arrangements to separate these ordered, concentrated particles from the clarified suspending phase[24,25].

Results for the removal of cells from suspensions in batch and USF-flow systems are shown in Table 1, which shows the diameters of cells tested, the active volume of the standing-wave region, the maximum removal and the concentration range over which effective removal has been achieved. It can be calculated from these values that a high efficiency is achieved when the biomass is in the range 0.01% to 1.0% w/w (wet weight of cells per unit weight of suspension). The result implies that the ability to form clumps at high biomass concentrations is an important aspect of harvesting of prokaryotes. The efficiency of sedimentation filters decreases, for a fixed flow rate, when biomass concentration increases above 15% w/v, as bands

of concentrated cells are more readily drawn out of the active region by viscous drag[2].

Batch systems may have a contribution to make to the clarification of suspensions for analytical purposes, as, for a fixed processing period, the residence time for which cells in a batch are exposed to ultrasound is longer than that for a flowing suspension. The clump-sedimentation pathlength can be short, so that separation times of the order required for centrifugal batch clarification of analytical samples[20] are feasible.

Ultrasonic enhancement of separation rates in biphasic aqueous systems

Highly selective separations can be achieved when cells or biomolecules partition rapidly between the two phases formed when high concentration of aqueous solutions of polymers such as poly(ethylene glycol) and dextran are mixed together[28,29]. The collection of the partitioned cells or solute involves coalescence and density-dependent movement of droplets to form two bulk phases. This process, which can take 30 min or longer at room temperature, is routinely accelerated by centrifugation. It has been shown that biphasic separation of *Saccharomyces cerevisiae* and *E. coli* can be achieved three times faster in a low-power ultrasonic standing wave than with centrifugation[3]. Differences between the mechanisms of development of two final bulk phases by ultrasound and by centrifugation include the very short (quarter-wavelength) distances that droplets need to move in order to form a layer in a sound field and the contribution of the interdroplet ultrasonic (Bjerknes) force[4] to coalescence. The ability to complete a partition separation more rapidly than centrifugation under low-ultrasonic-power conditions suggests that ultrasound has the potential required to facilitate bioanalytical applications of aqueous biphasic separations[30].

Ultrasonic-standing-wave enhancement of the rate and sensitivity of immunocoated-particle-agglutination assays and tests

Ultrasonic standing waves can also contribute to analytical biotechnology through a recently described technique that increases the rate and sensitivity of latex-agglutination tests (LATs). A LAT detects analytes through its ability to agglutinate antibody-coated latex microparticles[31]. These particle-based tests, as conventionally performed, can take times ranging from a few minutes to several hours, depending on the test format[32]. LATs remain popular because of their simplicity, speed and relatively low cost[33], although they can be, in some cases, as much as 20 times less sensitive (based on the lowest concentration of antigen detectable) than enzyme-linked immunosorbent assays (ELISAs)[34–36].

The improved performance of LATs in a standing wave follows from the increased local concentration of immunocoated particles that occurs when the acoustic forces drive the particles to pressure-nodal regions, and so particle-collision and -agglutination rates are dramatically higher than in the absence of ultrasound[32].

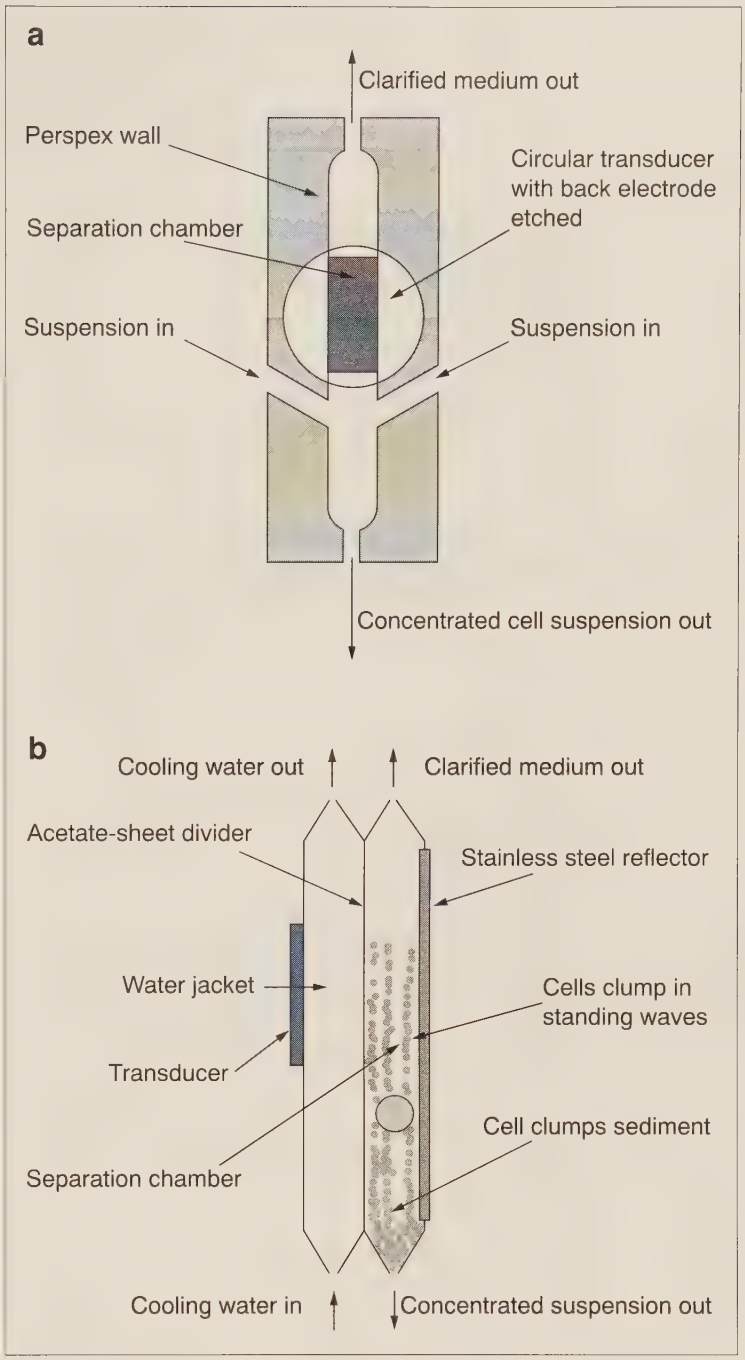

Figure 3

(**a**) Front view through a 7.5 ml sedimentation filter chamber. The dark-grey rectangular region represents the 1.0 × 1.5 cm electrode area on the back of a circular transducer. The cell suspension inlet is below the transducer. The suspension flows upwards, where cells are trapped in the standing wave. Cell clumps sediment out and are removed at the bottom of the filter, while the clarified liquid phase leaves at the top. (**b**) Side view: The acoustic path lengths through the cooling-water section and suspension are 1.45 and 1.25 cm, respectively. Reprinted with permission of the publisher from Hawkes, J. J., Limaye, M. S. and Coakley, W. T. (1997) 'Filtration of bacteria and yeast by ultrasound-enhanced sedimentation' *J. Appl. Microbiol.* 82, 39–47, Blackwell Science.

Ultrasonic concentration also increases the sensitivity of latex-agglutination tests, probably because particles are held close together for long times in the regions of high particle concentration. This increase (operationally defined as the ratio of the lowest antigen

Table 1. Parameters for the batch (B) or flow (F) removal of eukaryotes and prokaryotes from suspension in standing-wave devices

Cell(Refs)	Cell size (μm)[a]	System volume (ml)	Maximum removal (%)	Cell removal; over a concentration range (ml^{-1})
Saccharomyces cerevisiae[16]	5.0	5.5 (B)	97	>75% (10^7–10^8)
Escherichia coli[16]	1.3	5.5 (B)	72	>60% (10^9–10^{10})
Saccharomyces cerevisiae[18]	4.6	1.8 (F)[b]	99	>75% (10^6–4×10^9)
Escherichia coli[3]	1.3	1.8 (F)[b]	82	>60% (6×10^9–6×10^{10})
Hybridoma[26,27]	15	75 (F)	98	>95% (5×10^4–>8×10^5)

[a]Diameter of equivalent sphere.
[b]Volume is that of the resonator, not including tubing, entry and exit volumes and 'equilibration' flow requirement.

Table 2. Enhancement of latex-immunoagglutination assays in a standing wave compared to test-card agglutination[a]

Analyte(Refs)	Sensitivity enhancement
C-reactive protein[b;37]	×2560
Fungal antigens[5]	×256–×512
Escherichia coli 0157 antigen[38]	×1024
Neisseria meningitidis ACYW135[39]	×64
N. meningitidis B/*E. coli* K1[39]	×32
Streptococcus B[39]	×16
Streptococcus pneumoniae[39]	×16

[a]Enhancement is defined as the increase in dilution of antigen capable of a positive agglutination. The ultrasonic exposure times (1–5 min) were the same as those used for the test-card procedure.
[b]A nonspecific indicator of acute inflammation.

concentration detectable by eye on a test card to that detectable by microscopy following the ultrasonic procedure) in sensitivity is presented in Table 2 for a number of antigens.

Recent work showed quantitative relationships between the average area of coated-latex agglutinates formed in a standing wave and antigen concentration for the acute-inflammation marker, C-reactive protein[37], and for polysaccharide antigens of meningitis-causing bacteria[39]. These results open up the possibility of developing ultrasound-enhanced assays of analyte concentration.

Ultrasonic, dielectrophoretic and immunobead–magnetic-field separations

Ultrasonic radiation force effects on particles in suspension fall into the general area of physical forces on suspended particles. These include magnetic and dielectrophoretic forces and, of course, gravity and centrifugation. Ultrasound and dielectrophoresis currently rely on intrinsic differences in the mechanical or electrical[40] properties, respectively, between the particle (cell, droplet or coated bead) and suspending phase to exert a force on a cell or particle. Immuno-coated magnetic particles select a target in solution or suspension so that the manipulating magnetic force is exerted in a highly specific way on the bead–target complex. Magnetic beads find applications in the separation of cell (sub)populations[41] and of molecules, for example purifying DNA for PCR[42]. Dielectrophoresis, in which suspended cells in high-frequency (0.1–10 MHz) electric fields are drawn to and concentrate at arrays of electrodes, can fractionate cell populations on the basis of intrinsic differences in the frequency dependence of the force acting on different types of cells[40]; it should be noted that the sensitivity of latex-agglutination immunoassays can also be enhanced by dielectrophoresis[43]. The current lower size limits of microparticles (0.65 μm for single particles and 14 nm for particle ensembles)[44] that have been manipulated by dielectrophoresis are similar to those for ultrasonic fields [0.25 μm in intermediate volume suspension[35] to 40 nm in microchamber assemblies (J. J. Hawkes, pers. commun.)]. Dielectrophoretic separations require either very low volumes, to avoid heating in salt-containing suspensions, or desalination prior to separation in the field, but ultrasound is not thus constrained.

Future prospects

A high-performance USF capable of hybridoma retention is already commercially available (Sono floc, Biotech Inc., Richmond, BC, Canada), showing that the technology has now advanced beyond the 'proof of principle' stage. Smaller prototype sedimentation USFs, on a scale appropriate for analytical applications, have provided information on the dependence of USF efficiency on cell volume and concentration and on ultrasonic frequency and pressure amplitude, so that the design and construction of efficient filters for a range of different situations is now practicable. Current developments, which go beyond the sedimentation filter to flow systems that band cells ultrasonically and separate them from layers of clarified supernatant at the outflow, will extend the contribution of ultrasonic separation to analytical biotechnology. The technology may be scaled down to provide millimetre- and submillimetre-scale pathlengths for small-volume applications. Small-volume batch systems with longer retention times for cells in the active-standing-wave

region than flow systems, will also play a part in analytical separations. Enhancement of antigen detection by immunocoated-particle agglutination in a standing wave has progressed, in the case of meningitis-causing organisms, to the multicentre-clinical-trial stage. The identification of antibody-carrier particles that are suitable for generating high interparticle interactions in the sound field is expected to enhance antigen detection further, thus providing, *inter alia*, a simple technique for antigen monitoring at remote sites. Ultrasonic separation of cells, particles, droplets (biphasic separation) or gas bubbles may be appropriate where centrifugation is impractical or labour intensive, filters become blocked, the size of sample or the medium electrical conductivity is limiting in dielectrophoresis or heterogeneity of target cell surfaces precludes an immunomagnetic bead approach.

Acknowledgment

The author is grateful to J. J. Hawkes for useful discussions on the manuscript.

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The genetic reconstruction of BCG as a new immunotherapeutic tool

Michael A. O'Donnell

The bacillus of Calmette and Guérin (BCG), long appreciated for its role as a live vaccine for the prevention of tuberculosis, is undergoing a rebirth as a recombinant delivery vehicle for foreign antigens and bioactive proteins. Recombinant BCG causes long-lived specific humoral and cellular immunity and may ultimately prove to be a powerful and cost-effective new weapon against both infectious pathogens and certain cancers.

The development of an attenuated strain of virulent cow tuberculosis (*Mycobacterium bovis*) by Calmette and Guérin at the Pasteur Institute in 1921 as a vaccine for human tuberculosis (*Mycobacterium tuberculosis*) closely followed the overwhelming success of the vaccinia cowpox virus for the prevention of smallpox. This bacillus of Calmette and Guérin, commonly referred to as BCG, quickly became one of the most widely used live vaccines in the world, with more than 2.5 billion immunizations administered since 1948[1]. Its widespread use was the result of several advantageous features: as an immunogen, it provides long-term sensitization (5–50 years) with just a single inoculation, and multiple boosters are also possible; it can be administered as an oral vaccine; because it is not affected by maternal antibodies, it can be given at, or any time after, birth and boasts a very low incidence of serious complications, with a case-fatality ratio of 0.19 per million; its heat stability as a live but lyophilized product and low production costs (US$0.06 per dose) make it practical for use in many technologically and/or financially disadvantaged countries.

As a tuberculosis vaccine, BCG's effectiveness has been estimated at about 50%, but with wide variability of 0–80%, depending on the population studied[2]. Much of this variability has been traced to both genetic differences between populations and the presence of endogenous cross-reacting environmental mycobacteria, which are more prevalent in warmer areas. Because BCG vaccination leads to a positive tuberculin-skin-test reaction (PPD or Tine test), it severely impairs the ability to track the evolution of tuberculosis epidemics. For this reason, certain countries, notably the United States and Canada, have opted not to pursue a strategy of widespread immunization with BCG.

Owing to the clinical observation that patients with tuberculosis appeared to have a lower incidence of cancer, coupled with the apparent success of bacterial products (Coley's toxins) to promote cancer regression, BCG enjoyed a brief popularity as an anticancer agent between the 1940s and the 1960s. Although anecdotal successes were reported in widely different malignancies, such as lymphoma, lung cancer, malignant melanoma and even colon cancer, the development of more-efficacious treatments (chemotherapy and radiotherapy) eventually left BCG immunotherapy relegated to a limited role in specialized cancer-vaccine trials, most notably for melanomas[3]. A renaissance of BCG utility as a cancer agent has, however, occurred in the intercavitary treatment of superficial bladder cancer. With over two decades of substantiated clinical efficacy, BCG is now recognized as the single most effective adjuvant therapy for superficial bladder cancer, with complete-response rates exceeding 70% and long-term durability[4].

BCG as a target for genetic manipulation

The technological and commercial exploitation of the Gram-negative coliform bacteria, most notably *Escherichia coli*, through genetic DNA engineering has been due, in part, to the relative ease with which it can be manipulated, plus its rapid, 20-minute generation time. Until this decade, BCG had proved to be a particularly difficult bacterium to domesticate, in part owing to its much longer generation time of 20–24 hours and its resistance to many standard antimicrobial agents[5]. Furthermore, because of its thick, lipid-rich cell wall (the hallmark of its acid-fast staining behavior), BCG will not form individual colonies without the addition of a detergent to the growth medium.

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PII: S0167-7799(97)01134-7

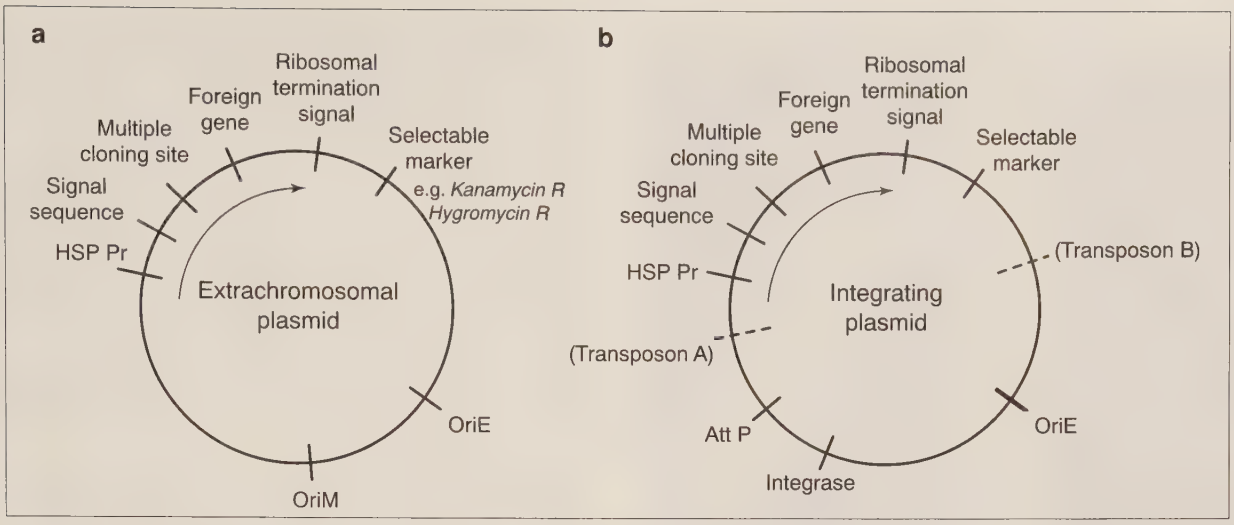

Figure 1

General schematic diagram of the extrachromosomal and integrating BCG vectors. (**a**) Extrachromosomal shuttle plasmids necessarily incorporate two origins of replication, specific for *Escherichia coli* (OriE) and mycobacteria (OriM). (**b**) Integrating vectors, by contrast, replicate in *E. coli* as plasmids, owing to the presence of OriE, but, because they lack OriM, they are only able to persist in mycobacteria by physical integration into the BCG genome. This is facilitated by flanking transposons that lead to random integration, or a phage attachment site (Att P) plus a gene encoding a phage integrase enzyme for site-directed integration. In both extrachromosomal and integrating plasmids, a strong dual-functional promoter such as the heat-shock promoter (HSP Pr) drives the expression of a foreign gene inserted into a downstream multiple-cloning site. An upstream *Mycobacterium*-specific signal sequence allows the export of the foreign product through the BCG cell wall. Transformed BCG (or *E. coli*) are commonly selected on the basis of resistance to antibiotics, usually kanamycin or hygromycin, although other selectable markers also exist.

Similarly, DNA transformation efficiencies by either conjugation or electroporation are often reduced relative to *E. coli* by over three orders of magnitude. Many of the prefabricated tools for bacterial genetic manipulation are also unsuitable for use in BCG because of the requirement for *Mycobacterium*-specific promoters, origins of replication and signal sequences, together with the high GC content of BCG DNA.

The construction of DNA vectors capable of shuttling between *E. coli* and mycobacteria has, coupled with the discovery of high-efficiency heat-shock promoters that work in both types of bacteria, greatly facilitated mycobacterial genetic manipulation[6,7]. In general, two types of vectors have been utilized: an extrachromosomal plasmid or an integrating variant (Fig. 1). Nonintegrating plasmids have the advantage of higher copy number (and thus, usually, higher recombinant-protein expression) and nondestructive alteration of the BCG genome. Although some loss of plasmid stability has been found in long-term cultures in the absence of continued antimicrobial selective pressure, significant numbers of plasmid-transformed BCG have been recovered even months after *in vivo* use[8,9]. First-generation integrating vectors, which rely on random integration into the BCG genome, for example from transposons, provide stable transcripts without the need for continued antibiotic selection. However, they may result in unexpected changes in BCG biology (including virulence), especially if multiple copies are introduced[10]. More recent developments permit site-specific integration of single gene copies into natural phage-integration sites, or gene replacement through homologous recombination[7,11].

Recombinant BCG as a foreign-antigen delivery vehicle

Although the techniques for mycobacterial genetic manipulation may potentially be applied to any species of *Mycobacterium*[5], the widespread and safe clinical use of live BCG as a tuberculosis vaccine has led to BCG being considered to be the most appropriate mycobacterial delivery vehicle for foreign-antigen immunization in humans. Mycobacterial products are well known for their efficacy as immune adjuvants. The activity of complete Freund's adjuvant, commonly used for experimental polyclonal-antibody production, for instance, relies on the presence of mycobacterial-cell-wall components. Slow-growing mycobacteria such as BCG engender strong, long-lived responses, partly owing to their ability to survive and replicate for several weeks or months in the host. In some cases, foci of live mycobacteria may persist for years in walled-off granulomas. As facultative intracellular pathogens, mycobacteria are particularly well-suited to eliciting cell-mediated immunity. Indeed, immune protection from mycobacterial infection has been shown to depend on the development of host T-helper-type-one (Th1) responses, which are characterized by the elaboration of a select cytokine profile including interleukins (IL) 2 and 12, and interferon gamma (IFN-γ)[12].

The expression of foreign antigens from BCG is currently undergoing rapid expansion and intense study. A multitude of different viral, parasitic, and bacterial antigens, including toxins, have been successfully expressed in recombinant BCG (rBCG) (Table 1). In some cases, the foreign antigen may account for up to

Table 1. Partial listing of foreign antigens from infectious pathogens expressed in recombinant BCG

Antigen	Refs
Human (and simian) immunodeficiency virus (HIV/SIV) proteins and/or epitopes(nef, gag, pol, env)	6,7,10, 13–16
Foot-and-mouth-disease-virus VP1 epitope	10
Measles-virus nucleocapsid protein	9
Plasmodium falciparum (malaria) circumsporozoite protein	17
Schistosoma mansoni (schistosomiasis) glutathione-*S*-transferase	18
Leishmania major (leishmaniasis) surface protease gp63	19
Mycobacterium leprae (leprosy) 18 kDa protein	20
Borrelia burgdorferi (Lyme disease) outer surface protein A	21
Streptococcus pneumoniae (pneumonia and otitis media) pneumococcal surface protein	22,23
Haemophilus influenzae (otitis media) peptidoglycan-associated protein	22
Tetanus toxoid ToxC	7

15% of the total BCG protein[23]. Studies in experimental rodents have shown that strong humoral (antibody) immunity can be elicited, usually persisting for months after a single inoculation[7,21]. Even higher antibody titres of longer duration have been achieved by additional rBCG inoculations[13,15]. Appropriate rBCG vaccines can also specifically activate all the major components of the cellular immune system, including delayed hypersensitivity, T-cell proliferation, Th1 cytokine production and major histocompatibility complex class I (MHC-I)-restricted cytotoxic-T-cell responses[6,7,9,14,16,19]. The induction of cell-mediated immunity is especially important for certain viral diseases, such as HIV, or diseases due to intracellular pathogens, such as *Leishmania* or *Mycobacterium leprae*, which are not efficiently cleared by neutralizing antibodies. Live antigen-expressing rBCG appears to be critical for providing strong, specific, cell-mediated immunity – both dead rBCG or live nonrecombinant BCG mixed with antigen are significantly less effective[7,24].

Complete protection against pathogen challenge has been achieved with rBCG in some cases, including leishmaniasis and pneumococcal infection[19,23], with partial protection in other cases, such as simian immunodeficiency virus (SIV) and measles[9,25], and no protection in others, for example, malaria and leprosy[17,20]. The specific immunogen, route of vaccine administration and manner in which the foreign antigen is expressed all play major roles in eliciting quantitatively and qualitatively distinct immune responses. For instance, intradermal or intraperitoneal inoculations of $2 \times 10^4 - 2 \times 10^6$ colony-forming units of rBCG expressing β-galactosidase produce similar systemic IgG responses, while intravenous administration has achieved high-level immunity with as few as 200 viable organisms[7]. Inoculation by the nasopharyngeal, but not the intraperitoneal, route with rBCG expressing the outer-surface-A protein of *Borrelia burgdorferi* (Lyme-disease pathogen) engendered long-lived, high-level mucosal immunity, characterized by high titres of IgA production[21]. Similarly, oral vaccination of mice with rBCG expressing the SIV nef protein resulted in strong, specific cytotoxic-T-cell responses from resident intestinal and mucosal T cells[26]. Thus, augmentation of immune defense mechanisms at local ports of entry appears possible with select modes of immunization with rBCG.

Vector constructs have been developed that enable foreign antigens to be expressed cytoplasmically or secreted outside rBCG as native proteins, chimeric soluble proteins or membrane-associated lipoproteins[15,23]. Although many foreign proteins expressed cytoplasmically in rBCG have been shown to lead to effective humoral and cellular immunity, several proteins are only effective immunogens when secreted outside the BCG bacterium[23].

Augmentation of rBCG immunogenicity by mammalian-cytokine expression

The conditions under which bacteria encounter host immune cells influence both the magnitude and polarity of the host immune response[12]. Using rBCG vectors employing mycobacterial signal peptides, rBCGs have been developed that secrete biologically functional proteins such as cytokines directly into the BCG microenvironment[27–29]. Certain cytokines, such as IL-2 and granulocyte–macrophage colony-stimulating factor, have been shown specifically to amplify the *in vitro* production of IFN-γ several times, which is characteristic of a polarized Th1 response[30]. *In vivo* testing of rBCG secreting human and mouse IL-2 in mice resulted in faster clearance of the BCG bacterial burden while, at the same time, causing stronger cellular-recall responses to BCG antigens[27,28]. Cytokine-producing rBCG thus has the potential to be a more effective antituberculosis vaccine. This strategy may facilitate the development of more potent live rBCG vaccines expressing foreign immunogens. By reducing the rBCG dose required for effective immunization, these vaccines may also be intrinsically safer. Preclinical studies demonstrating the important mechanistic role of BCG-induced cytokines for anticancer activity also suggest, in tandem with encouraging results from preliminary clinical trials of BCG-plus-cytokine mixtures, that this strategy may find a role in bladder-cancer and melanoma immunotherapy[30–33].

Elucidating the mechanisms of mycobacterial pathogenesis and drug treatment

Mycobacterial diseases caused by *M. tuberculosis*, *M. bovis*, *M. leprae* and the AIDS pathogen MAI (*Mycobacterium avium intracellulare*) remain serious worldwide health problems, accentuated by the

continuing emergence of strains resistant to multiple antibiotics. Transformation of mycobacteria with firefly-luciferase reporter constructs is accelerating the identification of antibiotic-resistant tuberculosis in clinical isolates, as well as facilitating rapid drug-screening programs for new candidate antimicrobial agents[34]. The insertion of other reporter proteins, such as β-galactosidase or jellyfish green fluorescent protein, is enabling researchers to study live mycobacterial pathogens from the standpoint of intracellular and whole-organ trafficking[35] (Fig. 2). These successful reporter systems will undoubtedly serve the further useful purpose of monitoring the fate of live rBCG vaccines once clinical trials are undertaken.

Potential hurdles to rBCG development and use

Theoretical, technical and regulatory hurdles are important factors in the short term for rBCG commercialization. From the foreign-antigen-delivery standpoint, it is unclear which immunogens or epitopes are likely to achieve the desired response of host immune protection. Certain antigens and bioactive proteins may rely on conformational structures that prokaryotes such as BCG are unable to produce; post-translational modification and disulphide linkages, for instance, are performed to a much more limited extent by mycobacteria[28]. In other cases, the immunogen of interest may be directly toxic to *in vitro* rBCG growth[7]. Some fear that prior sensitization to BCG by environmental exposure or deliberate vaccination with native or synthetic rBCG vaccines may eliminate rBCG too quickly for it to provide an adequate stimulus. A recent study, however, using rBCG expressing either β-galactosidase or HIV nef protein found only a modest (<50%) suppression of T-cell responses from BCG-primed animals, but greater high-specificity antibody production[13]. Similarly, objections to the loss of tuberculin-skin-test reactivity following therapeutic rBCG vaccination may be offset by the ability to engineer more-specific antigens that distinguish vaccination from pathogen exposure[36]. Finally, it is uncertain to what extent a live rBCG will encounter public and political resistance for direct human use. In this regard, an important precedent has already been set by the approval of genetically-engineered, attenuated, live oral cholera and typhoid vaccines, suggesting that the regulatory hurdles for rBCG are unlikely to be insurmountable. At least two major vaccine manufacturers have licensed the technology for rBCG commercialization.

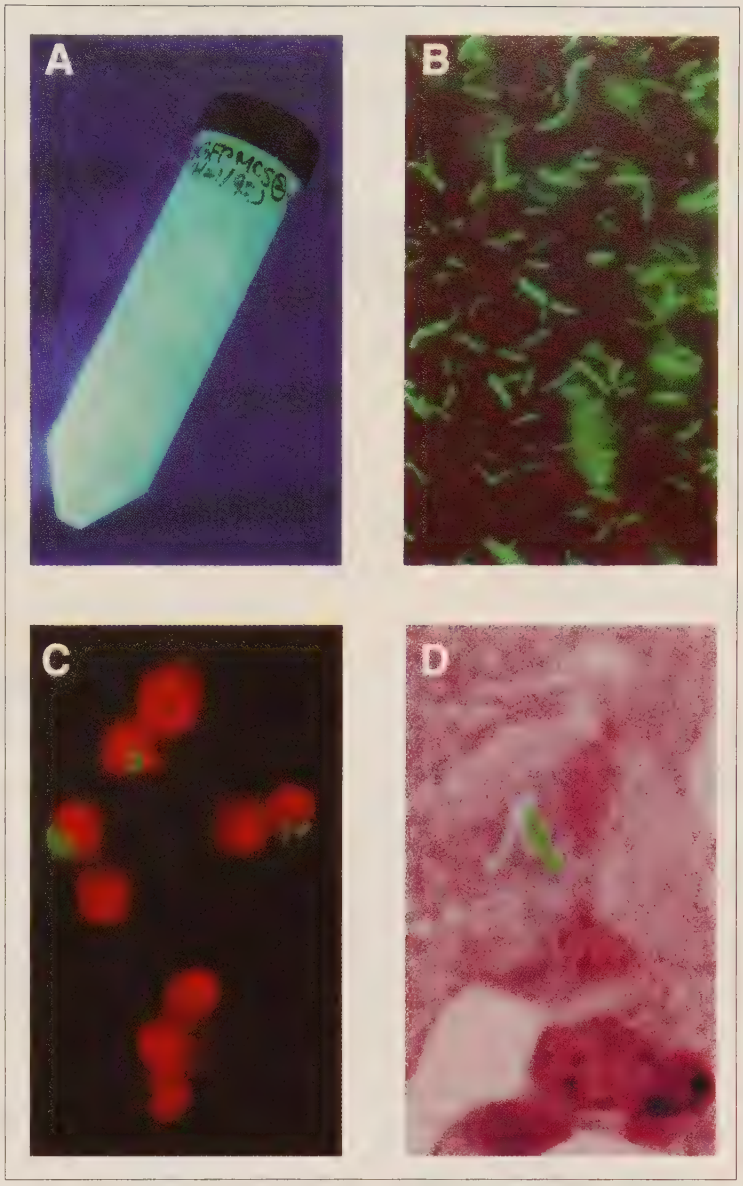

Figure 2

Green-fluorescent recombinant BCG (rBCG) as a means of following rBCG trafficking. (**a**) The brilliant fluorescence of a rBCG culture expressing the jellyfish green fluorescent protein (GFP) is readily apparent upon ultraviolet (UV) light exposure. (**b**) Visualization of individual bacilli of GFP rBCG under UV microscopy (200×). (**c**) Phagocytosis of GFP rBCG by a murine bladder-cancer cell line (in red), counterstained with ethidium bromide (100×). (**d**) Demonstration of GFP rBCG in a frozen section of mouse liver harvested one week after intravenous injection with GFP rBCG. The tissue was counterstained with haematoxylin and eosin and photographed under both visual and UV illumination (400×).

Future prospects for rBCG development

With the complete sequencing of the entire *M. tuberculosis* genome expected in the very near future, it is certain that the capacity for molecular-biological manipulation of BCG and its related cousins will expand greatly. Already, bicistronic or dual-promoter constructs have been developed that permit multiple linked protein expression, an advantage for the development of multivalent live BCG vaccines[22]. Furthermore, although the potential repertoire for expression of recombinant proteins in extrachromosomal plasmids may be physically limited, the large (>3 million base pair) BCG genome offers almost limitless opportunities for insertional incorporation. Regulated expression of recombinant proteins using inducible promoters or those active primarily after intracellular phagocytosis also appears likely[37]. To obviate the need for using potentially therapeutic antibiotics for rBCG selection, new markers for mercury resistance, phage resistance and sucrose sensitivity have been developed[38–40].

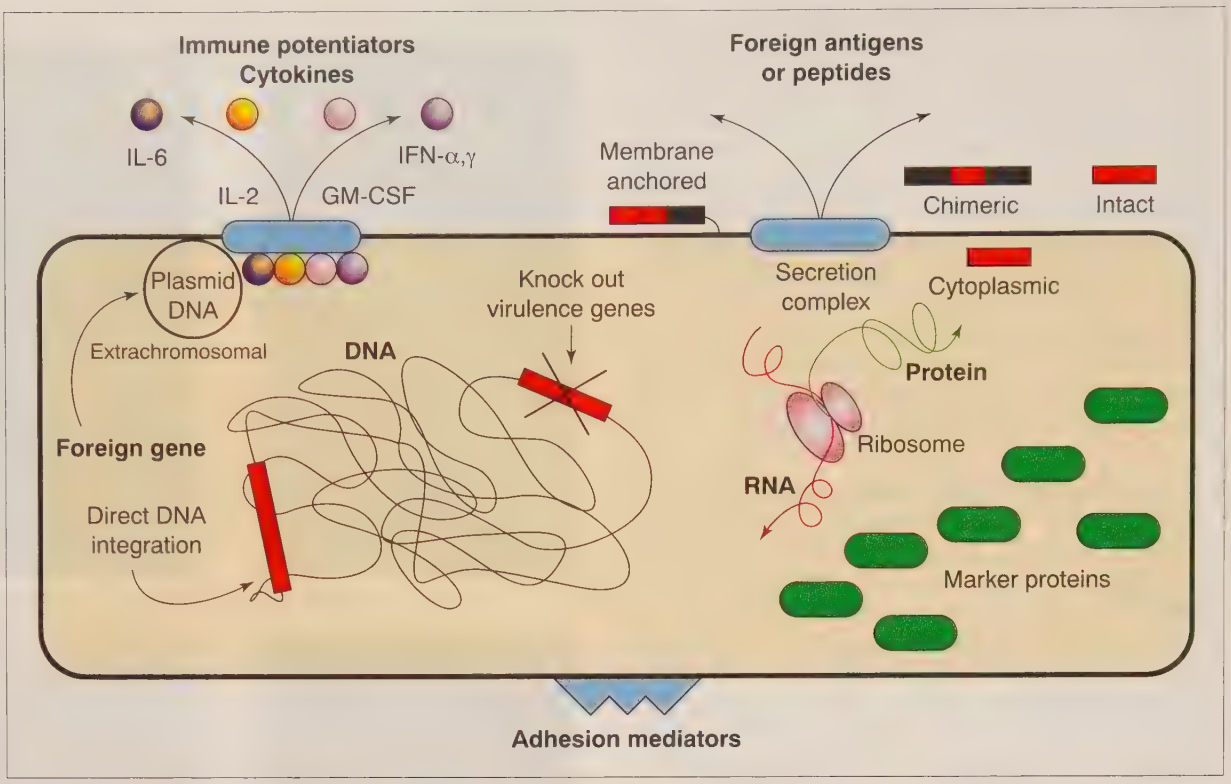

Figure 3

Some of the genetic modifications possible for recombinant BCG. rBCG can be engineered to produce a variety of foreign proteins, including immune potentiators, antigens, marker/reporter substances or even adhesion mediators. Without the addition of signal peptides, most foreign proteins would remain intracytoplasmic. However, by fusing the foreign gene to *Mycobacterium*-specific gene elements, the foreign protein can be secreted intact as a chimeric molecule or even as a membrane-associated product. Foreign genes can be maintained in BCG either in extrachromosomal plasmids or by direct genomic integration. Furthermore, homologous-recombination techniques may allow directed gene replacement to knock out native genes that code for undesirable properties, such as virulence.

Homologous-recombination techniques should permit the production of knockout rBCG strains that lack selected virulence features[11]. Similarly, genes may be inserted that sensitize rBCG to specific antimicrobial drugs, yielding a safer product. Modifications to surface membrane proteins may further restrict, or even direct, rBCG binding to specific tissue sites, where therapeutic and/or toxic products can be released intracellularly. A pictorial depiction of technologically accessible genetic alterations to BCG is provided in Fig. 3.

The medicinal use of the live BCG bacterium has survived decades of efforts to replace it with 'safer', structurally characterized, synthetic and nonviable alternatives. The fact that BCG is alive should not be viewed as a liability, however, but as a tremendous opportunity for future exploitation. Molecular techniques have already allowed this biological factory to be reengineered to produce foreign immunogens, bioactive cytokines and tracking proteins. Future prospects for multivalent and regulated protein expression appear secure. Although hurdles still remain before recombinant BCG vaccines reach the pharmacist's shelf, encouraging preclinical results suggest that clinical trials may soon be undertaken.

Acknowledgment

The author is supported in part by National Institutes of Health grant R29-CA64230.

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On-line and *in situ* monitoring of biomass in submerged cultivations

Lisbeth Olsson and Jens Nielsen

Biomass is one of the most important variables in submerged-cultivation processes and, in recent years, many different sensors have been introduced for on-line or *in situ* monitoring of this variable. This article provides an overview of the principles behind these sensors and discusses their application in both laboratory and industrial processes.

In bioprocesses involving living cells, the most central process variable is the concentration of the cell factory, that is, the biomass. Thus, for optimization and control of cultivations, it is important to quantify the efficiency of the cells, which is expressed as specific rates (e.g. moles product formed per unit biomass per unit time), and it is only through measurement of the biomass concentration that these specific rates can be extracted from cultivation data. The importance of biomass measurement is further illustrated by some quotations from the literature: 'Without doubt, the single most vital yet problematic value sought during fermentation is biomass concentration'[1]; 'During a fermentation process the determination of the viable and total cell count is one of the most important'[2]; 'The reliable on-line determination of the number of cells or the weight of the cells is still today the most important problem of measurement in biotechnology'[3]; 'Estimation of microbial biomass is by far the most important parameter of any fermentation process'[4]; 'Biomass concentration is of paramount interest to scientists as well as engineers in biotechnology'[5].

Despite the availability of a large number of experimental techniques for measuring biomass[6–8], there is, however, no method that is generally applicable for on-line monitoring of the biomass concentration in a process environment. In this article, we review some of the measurement techniques available and discuss their applications, including *in situ* sensors based on optical measurements (e.g. culture fluorescence and light scattering), measurement of the dielectric permittivity in cell suspensions or with ultrasound, methods based on on-line analysis, and software sensors (in which the biomass concentration is estimated from other measured culture variables, such as the rate of oxygen uptake and/or carbon-dioxide evolution).

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Biomass determination

The classical method for biomass determination is the gravimetric method, in which the biomass

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PII: S0167-7799(97)01136-0

Box 1. Standard operating procedure for biomass determination (dry weight) in a microwave oven

(1) Dry the filter (pore size 0.45 μm for yeast or fungus, 0.20 μm for bacteria) on a glass dish in the microwave oven on 150 W for 10 min, using the carousel. Put tissue paper or the like between the glass and the filter to avoid the filter sticking to the glass.
(2) Put the filter in a desiccator and allow to cool for 10–15 min. Weigh the filter.
(3) Filter the cell suspension through the filter and wash the cells with demineralized water.
(4) Place the filter on the glass dish again and dry in the microwave oven for 15 min at 150 W.
(5) Put the filter in a desiccator and allow to cool for 10–15 min. Weigh the filter.
(6) If more than 30 mg dry weight is present on the filter, the time in the microwave oven should be checked to ensure that it is long enough.

concentration is quantified by its dry weight. Today, this method is generally considered to be a reference method (Box 1 gives a standard operating procedure), and all other methods are typically compared with the dry weight. This method does, however, have a number of drawbacks and is therefore not really suitable as a reference method. For example, if there are other solids present in the medium, which is often the case for industrial media, the dry weight does not represent the biomass alone. Furthermore, the method measures the total biomass, that is, the sum of active cells and dead cells, which may not necessarily be the variable of interest. Normally, the active biomass is more important, for this is what determines the growth rate and productivity of the biomass. Despite the drawbacks of the gravimetric method and owing to the lack of an alternative, it is generally used as the reference method, and, in our discussion of the different methods, we will therefore also compare biomass measurements by other methods to the gravimetric biomass determination. It is, however, important to notice that some of the other methods may actually give a better measure of the active biomass than the gravimetric method and so correlate poorly with dry-weight measurements, especially over a wide range of operating conditions, such as the lag, exponential and stationary phases in a batch culture.

Because the different techniques supply different measures for the biomass concentration, a careful evaluation and comparison of sensors and measurement principles should ideally be carried out in cultivations with several sensors inserted into the bioreactor in order to obtain parallel readings. Alternatively, one can perform a 'reference cultivation' for each sensor, in which the environmental conditions experienced by the cells are well controlled and not changed from cultivation to cultivation. High-performance bioreactors are well suited to these kinds of investigations, because they combine the ability to perform reproducible cultivation experiments with that to monitor a large number of culture parameters and variables[9,10].

Requirements and limitations of *in situ* and on-line sensors

In situ and on-line sensors that will be used for biomass measurements should fulfil some general requirements[8]:

- reliability – with special emphasis on long-term stability and sterility (up to 20 days) in a pilot-plant environment;
- calibration – the response should be linear with biomass concentration in useful ranges (and, ideally, over a wide range), possess high resolution at critical concentrations (immediately following inoculation and at harvest), have a single calibration curve and the possibility of on-line calibration;
- interferences – there should be no interference from nonbiological solids and nonviable cells, little sensitivity to changes in operating conditions (agitation, aeration, dissolved-oxygen concentration, pH and temperature), no interference from ambient light and little sensitivity to changes in the medium composition;
- design – with special emphasis on the possibility of sterilization in place (SIP) and cleaning in place (CIP), the ability to insert the sensor through a standard 25 mm port and position it in a well-mixed zone of the bioreactor, a stable electrical signal, and allowing a similar design to be used in different applications (bacterial, yeast, fungal and animal-cell cultures).

None of the currently available sensors incorporates all these features, but the list may serve as a useful guide for future developments. Most of the commercially available sensors fulfill the requirements for SIP and CIP, and can be inserted into the bioreactor through standard 25 mm ports. However, very few sensors fulfil the requirement of few interferences and some are not sufficiently reliable, which is essential for their implementation in an industrial environment. Even though interference presents a serious problem, it is sometimes possible to take this into account in the signal analysis, but this requires a detailed characterization of the sensor and quantification of all possible interferences.

Measurement principles

In situ *optical sensors*

For off-line measurement of the biomass concentration, optical density (OD; see Glossary) is often considered to be a reference method. The OD of cultures in many culture media is correlated in a linear fashion with the biomass concentration, and measurement of the OD is attractive because it is fast and easy to perform. Linear correlations between the OD and the biomass concentration have led to the development of several *in situ* sensors for measuring the OD, and Table 1 gives an overview of some of the commercially available sensors. To ensure a wide dynamic range and good sensitivity, the emitted light should be focused and of high power (e.g. from a laser source), which is especially required for the measurement of backscattered light, because most of the scatter is in the forward direction. The wavelength measured in cultures of bacteria <3 μm in length should theoretically

Glossary	
Culture parameter	Variable that is controlled precisely at a set point during a cultivation and can therefore be kept constant (temperature and pH are typical culture parameters).
Culture variable	Variable that is not controlled during a cultivation, for example the concentrations of biomass or glucose during a batch culture.
Dielectrical permittivity	Consists of the capacitance (the ability to store electrical charges) and conductance (the ability to conduct electrical charges) of the subject.
High performance bioreactor	Bioreactor with a very low mixing time (<1 s), allowing the homogeneity of the medium to be assumed, with a high gas–liquid mass transfer ($k_L a$ >100 h^{-1}), with a large number of sensors and controllers that enable tight control of the culture parameters, and equipped with several sensors for on-line and/or *in situ* monitoring of culture variables.
***In situ* sensor**	Sensor that is inserted directly into the bioreactor and that can be sterilized in place.
OD	Optical density; that is, the reduction in transmitted light owing to light scattering in a sample. The transmitted light may be measured at different angles to the emitted light.
Off-line analysis	Analysis that is performed on a manually withdrawn sample.
On-line analysis	Analysis that is performed continuously (or at a very high frequency) and automatically on a sample.
SIP	Sterilizing in place.
CIP	Cleaning in place.

be in the visible region to give optimal sensitivity, whereas for larger organisms, infrared light is to be preferred[7]. Most of the commercially available sensors use a source that emits light in the lower-visible and/or near-infrared range (see Table 1), which has the advantage that most media have a low absorbance in this range (generally, the medium absorbance increases with decreasing wavelength).

The Cell-growth Probe (Wedgewood) and the FS1/FT1 (Monitek) are similar in design, measuring the forward light scatter. Both sensors are built with a notch (or indent) of a certain length – either 0.5 or 2 cm for the Cell-growth Probe and from 0.2 to 4 cm for the Monitek sensor. With a large notch, there is a longer light path, resulting in a larger sampling volume and thus greater sensitivity[8]. Both probes are very robust, but they are sensitive to agitation and aeration; it is possible to compensate for changes in the agitation rate with the FS1/FT1, but not for changes in the rate of aeration. In the Cell-growth Probe, a proprietary method is used to discriminate between the signal response caused by air bubbles and that caused by biomass.

In the Aquasant AS81/44 (Sulzer Biotech Systems) and Metler FSC402 (Ingold Electronics) sensors, the backscattered light is measured and therefore the sampling volume is large, which results in a high level of sensitivity. However, the interference from agitation and aeration is substantial, and, for filamentous-fungus cultivations, the signal is found to depend not only on the biomass concentration but also on the fungal morphology (C. L. Johansen, PhD thesis, Technical University of Denmark, 1993).

In the Max™ cell-mass sensor (Cerex), interferences from agitation, air flow, pressure and antifoam addition are minimized by passing the medium into an internal measurement chamber where the sample is degassed[8]. This gives a high correlation between the biomass concentration and the sensor signal up to about 60 g l^{-1}, but the complex design of the sensor may cause problems in an industrial environment, for example in connection with CIP.

Table 1. Overview of different *in situ* optical-density sensors

Sensor	Angle[a]	Wavelength (nm)	Light source	Manufacturer
Aquasant AS81/44	180°	900	Infrared light source	Sulzer Biotech Systems (Woodbury, NY, USA)
ASR Laser Turbidimeter	0°, 90° and 135°	780	Tungsten lamp	ASR Co. (Tokyo, Japan)
Cell-growth Probe	0°	750–980	Near-infrared lamp	Wedgewood Technology Inc. (Carlos, CA, USA)
Max™	180°	820–850	Laser diode	Cerex Corporation (Gaithersburg, MD, USA)
Metler FSC 402	180°	880	Light-emitting diode	Ingold Electronics Inc. (Switzerland)
Monitek FS1/FT1	0°	800–1100	Incandescent source	Monitek Technologies (Hayward, CA, USA)

[a]The angle specifies the measurement angle compared with the light source, that is, an angle of 0° represents the measurement of transmitted light and 180° represents the situation where backscattered light is measured.

Similarly, the ASR laser turbidimeter is based on the measurement of a degassed sample. The light source is a tungsten lamp, and it is possible to measure infrared and visible wavelengths through the use of filters[8]. Detection is performed at 0°, 90° and 135° from the angle of the emitted light, and this gives the ability to measure a wide range of cultivation systems as well as producing a wide dynamic range (0.1–450 g l^{-1}).

Culture fluorescence

Culture fluorescence was first applied by Duysens and Amesz in 1957[11] and, since then, this technique has been tested in a number of different cultivation systems[12]. Traditionally, it is NAD(P)H-dependent fluorescence that is measured (i.e. the fluorescence at 450 nm is measured after excitation at 360 nm), because there are *in situ* sensors commercially available for this purpose (Table 1). If the intracellular concentration of NAD(P)H is constant, the culture fluorescence may be directly correlated with the biomass concentration[13] and, initially these culture-fluorescence sensors were marketed as biomass sensors. However, good correlations between culture fluorescence and biomass concentration are only found for defined media and in conditions of balanced growth, that is, where there are no changes in the cellular composition. Interpretation of the NAD(P)H culture fluorescence is difficult, because it is affected by a number of factors[14–16] – cell concentration, cellular activity [i.e. intracellular level of NAD(P)H], geometry of the sensor, position of the sensor in the bioreactor, agitation and aeration rates, pH and temperature, medium viscosity, and background fluorescence.

A major problem with the application of NAD(P)H-dependent culture fluorescence results from the so-called inner-filter effects, which are caused by components in the medium that absorb light (at either 360 nm or 450 nm) and/or the presence of other fluorophores. The influence of inner-filter effects on the fluorescence signal is determined by several factors, including the length of the light path, the concentration of other fluorophores and absorbing compounds, and the geometry of the sensor[17,18]. The result of inner-filter effects has been illustrated by Nielsen *et al.*[19], who measured the culture fluoresence in fed-batch penicillin cultivations. They found that penicillin is a fluorophore, and that, when the penicillin concentration increased, the culture fluorescence decreased rapidly (Fig. 1). Therefore, the culture-fluorescence signal does not correlate with the biomass concentration.

From the above, it is obvious that culture fluorescence cannot be used for routine monitoring of the biomass during cultivations. The measurement principle is, however, interesting, because the sensors are reliable and reproducible, and many of the problems with the original sensors have been removed in a recently introduced sensor from Biobalance (Brøndby, Denmark). This sensor is based on a flashing light, which enables on-line compensation for background fluorescence. Furthermore, the sensor is very robust and therefore well suited to use in a process environment. However, the sensor does not overcome the problems with interference, but, with the introduction of multiple excitation–fluorescence measurements, it is possible to measure the fluorescence from several cellular fluorophores [tryptophan, pyridoxine, NAD(P)H, and riboflavin][15,16,20], and it is likely that a combination of these measurements with multivariate correlation will make it possible to produce a sensor that can give a good estimate for both the biomass concentration and the biomass activity.

Capacitance-based instruments

Measurement of dielectric properties is one of the latest contributions in the determination of biomass; these consist of capacitance (the ability to store electrical charge) and conductance (the ability to conduct electrical charge)[21]. Assuming that the physiological state of the cells is unchanged, the capacitance of a culture is a function of the total cell volume, which depends on the concentration, viability and size of the cells. The effect of the medium conductance must be calibrated before the cell concentration can be determined. Alternatively, the biomass measurements can be based on a dual-frequency technique, in which the capacitance measurement is performed at two frequencies, at one of which only the medium contributes to the reading, and at the other both the medium and the cells contribute[22]. Linear correlations with dry-weight measurements have been shown for *Escherichia coli* (0–37 g l^{-1}), *Saccharomyces cerevisiae* (0–40 g l^{-1}) and *Perilla frutescens* (0–10 g l^{-1})[21].

The commonest capacitance sensor is the ABER Biomass Monitor (previously called the Bugmeter), which is restricted to measuring cultivation media with conductivities below 20 mS[23]. The instrument works in the range 0.1–10 MHz, which is the same range within which cells act as capacitors. Changes in aeration and agitation interfere with the readings, as the permittivity of gas bubbles is lower than that of the medium[23]. Additionally, it has been shown that extraneous radio frequencies will influence the capacitance reading, and Matanguihan *et al.*[21] have shown that capacitance sensors can suffer from signal fluctuation. The problem with possible fouling of the sensors has, in this case, been solved by introducing periodic electrochemical cleaning[8].

One disadvantage with this method is that the capacitance changes with the physiological state of the culture. Anything that disturbs the flow of ions on the cell surface or alters the composition, structure or permeability of the membrane can affect the capacitance[24]. Dry-weight determination and capacitance measurement correlated well during a batch fermentation with *S. cerevisiae* but, when the batch cultivation reached stationary phase, the capacitance measurement decreased, probably owing to a decrease in the cell size[21]. The capacitance depends on the presence of an intact cell membrane, and so lysed cells do not contribute to the measurement[22]. In another study, the dry-weight and capacitance measurements were

correlated during a batch cultivation of *Saccharomyces uvarum*, and it was found that the correlation between the two methods was poor[21]; the capacitance did, however, correlate well with the number of viable cells.

Fehrenbach *et al.*[23] investigated the use of the Biomass Monitor in cultivations of *S. cerevisiae, Pichia pastoris* and *Streptomyces virginiae* in relation to other biomass measurements. The investigations were performed in both defined and complex media and in bioreactors from 20 to 2000 l. Generally, they achieved poor correlations between the Biomass Monitor and the other methods at the start of the cultivations but, in the exponential-growth phase, reasonable correlations were found between conductivity and the other estimates of biomass. The authors concluded that the correlations could be considered sufficiently reliable for industrial applications, but doubtful for scientific applications.

On-line analysis

On-line analysis is based on automatic sample withdrawal and measurement, and there have been some reports of the implementation of different on-line techniques. Reuss *et al.*[25] developed an automated filtration system that enables automatic determination of the biomass concentration. The equipment is especially suited to filamentous-fungus cultivations, because these organisms build up a large filter cake. Using such a system, it is also possible to obtain information about the density of the cells, which may give valuable information about the degree of cellular differentiation in fungal cultures[26]. These systems do, however, require a large sample volume and are therefore not suitable for high-frequency monitoring of laboratory bioreactors.

Another approach to on-line biomass monitoring is the application of flow-injection analysis (FIA) or sequential-injection analysis (SIA)[27–30]. In these methods, the sample is withdrawn through a syringe and pumped to the analyser by a peristaltic pump; all the analysers that have been described are based on the measurement of the OD in the visible-light range. FIA and SIA have the advantage of being fast and easy to implement, and the dynamic range can be wide if on-line sample dilution is included as a part of the analysis[28,30]. Furthermore, as measurements are made on a withdrawn sample, it is possible to recalibrate the analysers during a cultivation process. Despite their advantages, these methods require substantial improvements – especially in terms of robustness – before they will be ready for implementation in a process environment. Furthermore, they have only been demonstrated to work for unicellular cultures.

Ultrasonic measurements

It has been found that the biomass concentration can be determined from the difference between the velocity of sound in the suspension and in the cell-free solution. The technique anticipates that the density of the medium and the density and compressibility of the cells remain constant. Reasonably linear calibration curves have been achieved within a range of 0–10 g l^{-1} for bacteria, yeast and filamentous fungi[31]. Ultrasonic measurements have been shown to be applicable over a broad measurement range; a linear calibration curve was determined for *E. coli* in an OD range of 0–250[32]. However, the need for a cell-free reference requires either the withdrawal of the sample or *in situ* measurement of the background signal. The changes in the attenuation of the ultrasound can also be used as a measure of biomass concentration. The attenuation is sensitive to changes in the viscosity of the medium. Owing to the vibrational nature of the device, it is, in principle, self-cleaning. General problems that arise with these methods include the influence of gas bubbles, changes in medium composition during the process and the fact that the measurements have been shown to be sensitive to temperature fluctuations[8,31,32].

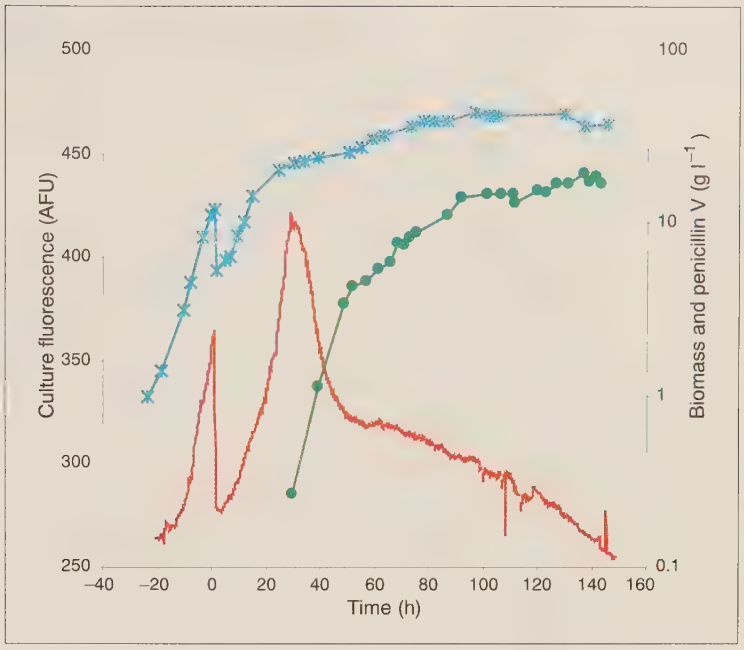

Figure 1

Measurement of the NAD(P)H-dependent culture fluorescence during a fed-batch penicillin cultivation with an *in situ* sensor (Mettler FSC402, Ingold AG) (red). The biomass concentration (blue; determined as dry weight) and the penicillin concentration (green) are also shown. The fed-batch process was preceded by a batch cultivation (from –20 h to 0 h) for which the culture fluorescence and the biomass concentration are also shown. The rapid increase in the culture fluorescence during the first 30 h of the fed-batch cultivation is caused by the increase in the biomass concentration, whereas the rapid decrease after 30 h is caused by interference from penicillin. Data taken from Ref. 19.

Software sensors

Software sensors are based on estimation of the culture variable of interest from measurements of other culture variables using a mathematical model. This model may be a simple algorithm based on elemental balances or a nonlinear model, such as a neural-network model. Several different software sensors for biomass estimation have been described, but here we will illustrate this principle with two applications.

Petkov and Davis[33] estimated the biomass concentration using a software sensor in batch cultivations of *Corynebacterium glutamicum*. Carbon dioxide and oxygen were measured on line, and, with the aid of the respiratory quotient, the fraction of the total oxygen utilization rate required for cell maintenance and growth was determined relative to that needed for L-lysine synthesis. The biomass concentration could then be estimated from the modified oxygen utilization rate by integrating the oxygen balance for cell growth and maintenance. With this simple algorithm, the biomass concentration was estimated with a maximum relative deviation of 8%. Di Massimo-Peel *et al.*[34] demonstrated the successful application of an artificial neural network as a biomass estimator in an industrial penicillin cultivation and in a cultivation of the filamentous fungus *Fusarium graminearum*. This estimation was based on the measurement of the carbon-dioxide-evolution, alkali-addition and dilution rates. The neural network was trained by analysing a set of cultivation data, after which it demonstrated a predictive power accurate enough to be used for control purposes. It was also shown that the biomass could be accurately estimated even though the carbon-dioxide measurement broke down. This is because several variables were used to estimate the biomass.

Concluding remarks

Biomass concentration is the most important variable in cultivation processes and, as described here, many different techniques have been developed for on-line or *in situ* measurement of this variable. Generally, an *in situ* sensor is to be preferred because it may result in an almost continuous measurement of the concentration, but there is currently no sensor that stands out as being the definitive biomass sensor. The optical sensors may produce results that correlate with the biomass concentrations in some systems, but, especially in high-density cultures, calibration is a problem. Furthermore, different designs may have to be used for different applications, such as microbial and mammalian-cell cultures. Probably the best available *in situ* sensor is the Biomass Monitor (Aber Instruments), which is based on measurements of the dielectric permittivity. However, as for the optical sensors, calibration is difficult and the current design is not sufficiently robust for implementation in an industrial-process environment. As has been mentioned, multivariate analysis (that is, analysis based on multiple sensor signals) may be an attractive approach, and the future will probably bring novel sensors based on several of these measurement principles, integrated with chemometric analysis. These sensors may be designed to fulfil most of the criteria specified above and may, therefore, find wide application in both research laboratories and industrial processes. In the development of such novel sensors, it is important, however, to have knowledge about the system, both chemical and biological, that one wants to measure.

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Directed evolution of enzyme catalysts

Olga Kuchner and Frances H. Arnold

Directed enzyme evolution has emerged in the past few years as a powerful alternative to rational approaches for engineering biocatalysts. Prerequisites for successful directed evolution are functional expression in a suitable microbial host, a rapid screen for the desired feature(s) and a well-thought-out working strategy for navigating protein landscapes. The rapidly growing body of literature on enzyme evolution *in vitro* includes techniques for creating and searching combinatorial enzyme libraries, as well as several successful examples of different evolutionary strategies being used.

Enzymes' efficiency and precision are unmatched by most conventional industrial catalysts. However, naturally occurring enzymes often lack features necessary for commercial applications. Although protein chemists continue to elucidate the relationships between the sequence, structure and function of proteins, the extensive knowledge that is necessary for the application of rational engineering approaches is available for only a tiny fraction of known enzymes. Directed evolution has proved to be useful for modifying enzymes in the absence of such knowledge.

Directed enzyme evolution generally begins with the creation of a library of mutated genes. Gene products that show improvement with respect to the desired property or set of properties are identified by selection or screening, and the gene(s) encoding those enzymes are subjected to further cycles of mutation and screening in order to accumulate beneficial mutations. This evolution can involve few or many generations, depending on how far one wishes to progress and the effects of mutations observed in each generation.

The main requirements for successful directed evolution are (1) the functional expression of the enzyme in a suitable microbial host, (2) the availability of a screen (or selection) sensitive to the desired properties and (3) identifying a workable evolution strategy. The vast majority of possible evolutionary paths lead to poorer enzymes; the strategic challenge is to identify a path that will result in the improvement of the desired feature(s). The number of possible variants increases rapidly with the size of the enzyme and with the number of amino acids that are allowed to vary simultaneously (Box 1). Even for a small protein, an impossibly large number of variants can be generated when multiple mutations are introduced. Because most mutations are deleterious[1,2], the chances of identifying improved enzymes in libraries containing large numbers of mutations are very small indeed. Therefore, the mutation rate must be tuned to the power of the screen or selection[3]. We will comment on some strategies for creating and searching libraries of enzyme variants. In addition, selected theoretical and experimental investigations of protein 'fitness landscapes' that may be applied to optimize these strategies will be introduced.

Interest in engineering enzymes by directed evolution has grown significantly in the past few years. Table 1 summarizes some key examples of directed enzyme evolution in which multiple generations of mutagenesis and screening or selection have been performed[4–13].

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The evolution of thermostability

Improvements in stability, particularly thermostability, are commonly sought by protein engineers. In industrial processes, high temperatures impart such benefits as increased substrate solubility, decreased viscosity of the medium, lower risk of microbial contamination or higher rates of concurrent non-enzymatic reactions. In the large number of random-mutagenesis experiments that have targeted thermostability, single beneficial mutations typically increase the melting temperature (T_m) of the enzyme by 1–2°C[14–18]. Much larger changes are possible, but rare. Pjura *et al.*[15] identified eleven single mutations that increased the thermostability of T4 lysozyme by 0.8 to 1.4°C. Haruki *et al.*[14] found eight mutations that increased the stability of *Escherichia coli* ribonuclease HI; seven impart a ΔT_m between 0.3°C and 4.2°C, while

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PII: S0167-7799(97)01138-4

Box 1. The number of possible variants of a protein that can be created by introducing *M* substitutions simultaneously over *N* amino acids is 19^M [$N! / (N - M)!\ M!$]

		Sequence length = *N* (number of positions targeted)		
		5	10	200
Number of amino acids changed simultaneously	1	95	190	3 800
	2	14 440	16 245	7 183 900
	3	68 590	823 080	9 008 610 600
	4	651 605	27 367 410	8 429 807 368 950
	5	2 476 099	623 976 948	6 278 520 528 393 960
	6		9 879 635 010	3 876 986 426 283 270 000
	7		107 264 608 860	2 041 510 281 040 010 000 000

This table lists the number of variants that could be made if every possible amino acid substitution were accessible (for example, by codon-synthesis methods). If point mutations are introduced randomly at the DNA level, the accessible sequence diversity is limited. On average, only 5.7 different amino acids are accessible by single-base-pair changes in a codon. Furthermore, the number of clones that must be screened to see a given number of unique amino-acid-level variants must increase to account for wild-type variants arising from the degeneracy of the genetic code. To have 95% confidence that a given number of amino-acid-level variants has been sampled in a screen or selection, the number of unique clones must be multiplied by a factor of approximately ten.

The feasible upper limit for screening with a high-throughput assay is indicated by the darker gray area. The upper limit of feasibility for selection in microbial hosts is indicated by lighter gray. Neither screening nor selection allow exhaustive searches of libraries containing multiple mutations distributed over long sequences.

one amino acid substitution (which is thought to fill a cavity in the protein's interior) yielded a full 7.8°C increase. T_m values reported for double and triple mutants[19,20] appear consistent with the generalization that the free-energy effects of individual stabilizing mutations are roughly additive[21].

Significantly larger increases in thermostability resulting from single amino acid substitutions have also been reported. Joyet *et al.*[22] combined a stabilizing mutation they had identified using sequence comparisons between mesophilic and thermophilic versions of α-amylases with a new mutation generated by random mutagenesis to raise the T_m of barley α-amylase by 11°C and increase its half-life at 90°C nine- to tenfold. In an early directed-evolution experiment, Liao *et al.*[4] found two amino acid substitutions in the antibiotic-resistance enzyme kanamycin nucleotidyltransferase that resulted in a 15°C increase in T_m. They performed two sequential rounds of mutagenesis and selection by growing *Bacillus stearothermophilus* expressing the kanamycin-nucleotidyltransferase gene (*kan*) in a chemostat and identified one mutation in each round. Remarkably, the same two amino acid substitutions had also been found by Matsumura and Aiba[23], who found the two mutations in a single clone after a single round of random mutagenesis and screening. Stable variants that imparted kanamycin resistance were identified by infecting *E. coli* cells with *kan*-transducing phage and incubating at increasingly higher temperatures.

Table 1. Summary of key directed-enzyme-evolution experiments utilizing sequential generations of random mutagenesis and recombination with selection or screening.

Enzyme	Altered property	Mutagenesis method	Screened or selected	Refs
Kanamycin nucleotidyltransferase	Thermostability	Mutator strain	Selected	4
Subtilisin E	Activity in organic solvents	Error-prone PCR	Screened	5,6
β-Lactamase	Total activity and substrate specificity	DNA shuffling	Selected	7
Subtilisin BPN'	Stability	Cassette	Screened	8
Paranitrobenzyl esterase	Substrate specificity and activity in organic solvents	Error-prone PCR and recombination	Screened	9
Thymidine kinase	Substrate specificity	Cassette	Both	10
β-Galactosidase	Substrate specificity	DNA shuffling	Screened	11
Arsenate detoxification pathway	Arsenic resistance	DNA shuffling	Screened	12
Paranitrobenzyl esterase	Substrate specificity and activity in organic solvents	Error-prone PCR and DNA shuffling	Screened	13

Various strategies have been devised for combating the difficulties encountered in screening highly stable enzymes, which include temperature limitations of the host organisms and the awkwardness of assaying for activity at very high temperatures. Several groups have devised schemes to select for thermostability in thermophilic organisms[4,14,16,24]; some contend that their systems can be readily adapted to the evolution of other proteins, but each of these experiments was performed using an enzyme model system (kanamycin nucleotidyltransferase[4], 3-isopropylmalate dehydrogenase[16,24] or ribonuclease HI[14]) that is highly amenable to selection – that is, the functional enzyme is critical to the survival and growth of the host. Four groups[14,16,22,24] created temperature-sensitive variants of the enzymes they wished to stabilize and subsequently screened for intragenic suppressor mutations that compensated for the loss of stability. This approach offers the advantage that screening can be performed at a lower temperature than that needed to deactivate the wild-type enzyme. However, it poses the risk of identifying mutations that compensate for the initial destabilization but do not confer an advantage to the wild-type sequence. In the experiments reported by Joyet *et al.*[22] and Kotsuka *et al.*[16], the single second-site suppressor mutations also increased the thermostability of the wild-type enzyme. Haruki *et al.*[14] identified eleven second-site suppressor mutations, eight of which were also beneficial in the wild-type enzyme.

Screening may offer a more efficient and generally applicable approach to evolution of thermostability than selection[25]. Using replica plates during screening avoids subjecting host organisms to the temperatures needed to deactivate thermostable enzymes. A typical result of screening paranitrobenzyl-esterase variants for enhanced thermostability is shown in Fig. 1. In this example, a significant fraction of the variants in each 96-well plate appear more thermostable than the parent. Eleven amino acid substitutions, accumulated during sequential mutagenesis and screening, have resulted in a 14°C increase in the esterase T_m (L. Giver, P. O. Freskgard and F. H. Arnold, unpublished).

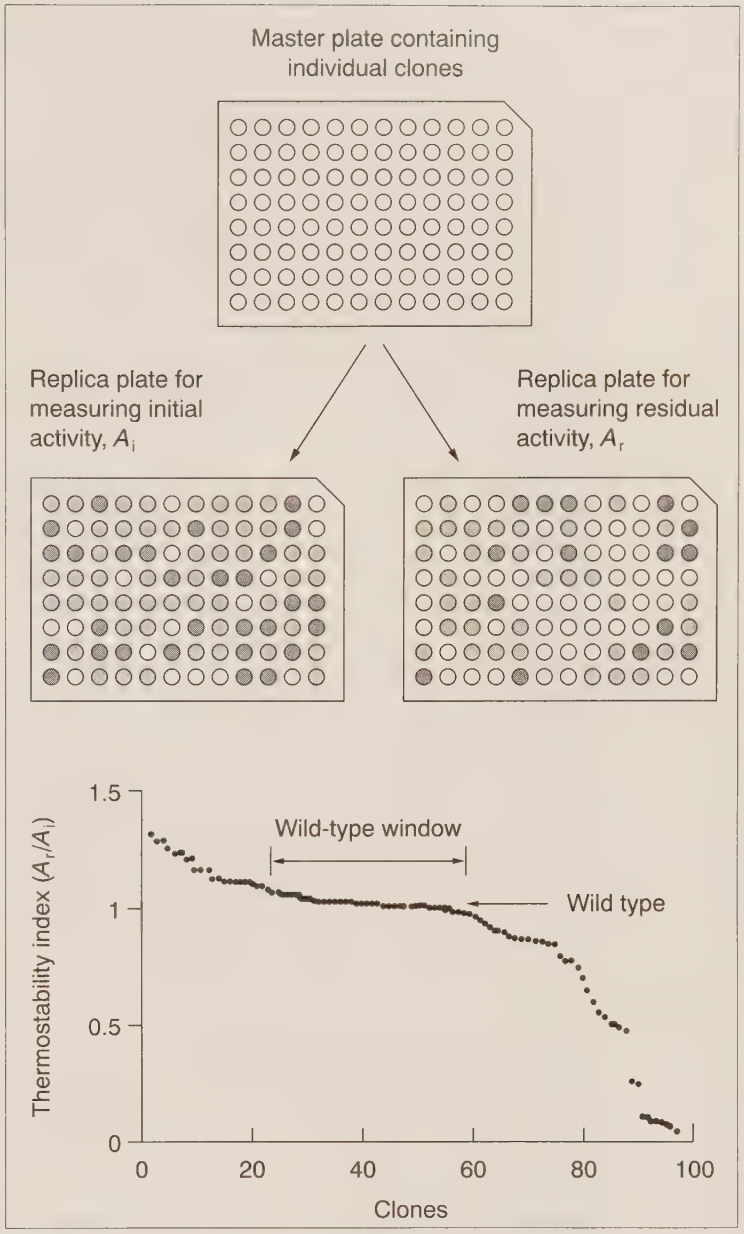


Figure 1

A simple screen for enzyme thermostability based on catalytic activity (*A*) before and after incubation at high temperature. Transformants of a randomly recombined paranitrobenzyl-esterase library were picked and grown in 96-well plates. Initial activity (A_i) and residual activity (A_r) after incubation at the T_m of the parent were measured in two separate replica plates using a microtitre plate reader. A_r/A_i values are sorted and plotted in descending order to show the local fitness profile. Useful parameters derived from this profile include the frequency and fitness of improved clones, the frequency of inactive clones (related to the point-mutagenesis rate) and the size of the wild-type window. Accumulation of mutations over five generations of random mutagenesis and screening has increased the T_m from 52 to 66°C (L. Giver, P. O. Freskgard and F. H. Arnold, unpublished).

The evolution of stability or activity in artificial environments

The use of organic solvents can facilitate solubilization of a substrate or enhance the rate of one reaction over another. Naturally occurring enzymes, however, generally lose activity in organic solvents or aqueous–organic mixtures, even when the native structure remains stable. In an early application of directed evolution, the protease subtilisin E was subjected to sequential generations of random mutagenesis and screening[5] to yield a variant that is active in 60% dimethylformamide (DMF) almost as efficiently as the wild type is in water, an increase of nearly 500 times in the k_{cat}:K_M ratio (Ref. 6). More recently, both sequential random mutagenesis and gene recombination were used to evolve paranitrobenzyl esterase for deprotection of an antibiotic synthetic intermediate in aqueous DMF. The total activity in 30% DMF was increased more than 100 times[9,13].

The first use of random mutagenesis to improve the activity of a mesophilic enzyme at low temperatures was recently reported by Kano *et al.*[26], who screened random variants of subtilisin BPN′ for activity at 10°C. The k_{cat} value of one improved variant was close to that of the wild-type enzyme, and decreased to the same extent as the k_{cat} of the wild-type enzyme in response to a shift from 25°C to 5°C. However, its K_M

value decreased much more sharply than that of the wild type in response to the same temperature shift, resulting in a 10% increase in hydrolytic activity at 10°C and a 30% increase in activity at 1°C. The stability of the evolved enzyme to higher temperature was unchanged. Many enzymes from psychrophilic organisms are more thermolabile than their mesophilic counterparts, and it has been claimed that the flexibility required for activity at cold temperatures inherently limits stability at moderate temperatures. Directed evolution should be able to answer the question of whether psychrophilic enzymes indeed have such intrinsic limitations to thermostability. It may be that these properties have simply not been subjected to evolutionary pressures.

Changing or eliminating an enzyme's requirements for metal ions can be useful in laundry detergents, which commonly contain chelating agents that maintain low free metal-ion concentrations and often use proteases that depend on metal ions for their activity or stability. Bryan and co-workers[8] evolved a subtilisin that is stable in the absence of calcium. After first deleting the enzyme's calcium-binding loop (creating a highly destabilized subtilisin), they randomly mutated ten amino acids that had interacted with that loop and were thus assumed no longer to be optimized for stability in the modified enzyme. The half-life of the evolved subtilisin in low free-calcium concentrations is 12.5 times higher than that of wild-type subtilisin BPN′. Additional rounds of mutagenesis and screening have resulted in an even more stable calcium-independent subtilisin (P. Bryan, pers. commun.).

The evolution of substrate specificity and increased activity on novel substrates

Modifying enzyme substrate specificity represents a critical frontier for adapting enzymes to industrially desirable reactions. A number of random-mutagenesis and directed-evolution experiments have demonstrated that substrate preferences can be evolved. In many of these experiments[10,11,27–30], the increased catalytic efficiency of improved variants resulted from a decrease in K_M. For example, Sidhu and Borgford[29] found variants of *Streptomyces griseus* protease B with dramatically increased activity towards peptides with methionine at the P1 binding site; these variants displayed practically no change in k_{cat}. In other cases, such as the modification of a T7 RNA polymerase to function as a DNA polymerase[31], changes in both k_{cat} and K_M are observed. Beuve and Danchin[27] demonstrated a change in primary substrate specificity of adenylate cyclase by isolating a variant that exhibited a four-and-a-half-fold increase in guanylate-cyclase activity, while retaining only 4% of the wild-type adenylate-cyclase activity. A second mutation restored the wild-type adenylate-cyclase activity and increased guanylate-cyclase activity tenfold relative to the wild type, in effect broadening the enzyme's substrate specificity. An *E. coli* β-galactosidase evolved *in vitro* by Zhang *et al.*[11] to have greater activity towards fucose substrates showed a twofold decrease in k_{cat}, but a 20-fold decrease in K_M.

The directed-evolution experiments reported by Zhang *et al.*[11] and Moore and Arnold[9] are among the very few in which random mutagenesis was not restricted to the amino acids believed to constitute the substrate-binding site. Zhang *et al.* performed seven rounds of DNA shuffling and screening using chromogenic substrates. Six amino acid substitutions were accumulated during the evolution, three of which are located in domain 3 (residues 334–627), which contains most of the substrate-binding pocket. The other three amino acid changes are not located near the binding pocket or active site of the enzyme. The paranitrobenzyl esterase that was evolved for activity towards a novel antibiotic substrate in organic solvents[9] initially displayed only low activity towards this substrate. The increased specific activity achieved by directed evolution reflected adaptation towards the new substrate as well as the solvent environment, and yet none of the mutations accumulated in the evolved enzyme were in direct contact with the substrate: some were as far away as 20 Å. It is clear that limiting the search for possible solutions to residues that line the substrate-binding site will overlook important beneficial mutations.

Two groups have evolved glutathione transferases to create more efficient drug-detoxification enzymes. Gulick and Fahl[32] isolated a variant of glutathione-*S*-transferase that confers ninefold-greater resistance to an alkylating agent used in cancer chemotherapy by selecting for catalytic efficiency. Using phage display to identify glutathione transferases with improved binding to several electrophilic substrates, Widersten and Mannervik[33] failed to isolate any variants more active than the wild-type enzyme. One disadvantage of screening for binding rather than catalysis is that transition-state stabilization, the phenomenological definition of catalysis, involves mechanisms besides transition-state binding. Reinforcing transition-state binding beyond an optimum level may restrict the motion of the enzyme necessary for conformational changes during catalysis, as shown by the small increase in binding affinity that is used for transition-state stabilization compared with the total increase in binding affinity acquired by catalytic antibodies during somatic hypermutation[34].

The evolution of enantioselectivity

Matcham and Bowen[35] reported the use of directed-evolution methods to enhance the enantioselectivity of an *R*-transaminase. Transaminases have been used to produce chiral amines either directly from the precursor ketone or by resolution of the racemic amine through conversion of the amine with incorrect stereochemistry back to the ketone. Most transaminases convert ketones to the corresponding amine with a greater-than-99% enantiomeric excess, but one *S*-selective transaminase converted a β-tetralone to the desired amine with only a 65% enantiomeric excess. 10 000 randomly mutagenized colonies were screened to yield ten variants that increased the enantiomeric excess of this conversion to 80–94%. Some of the

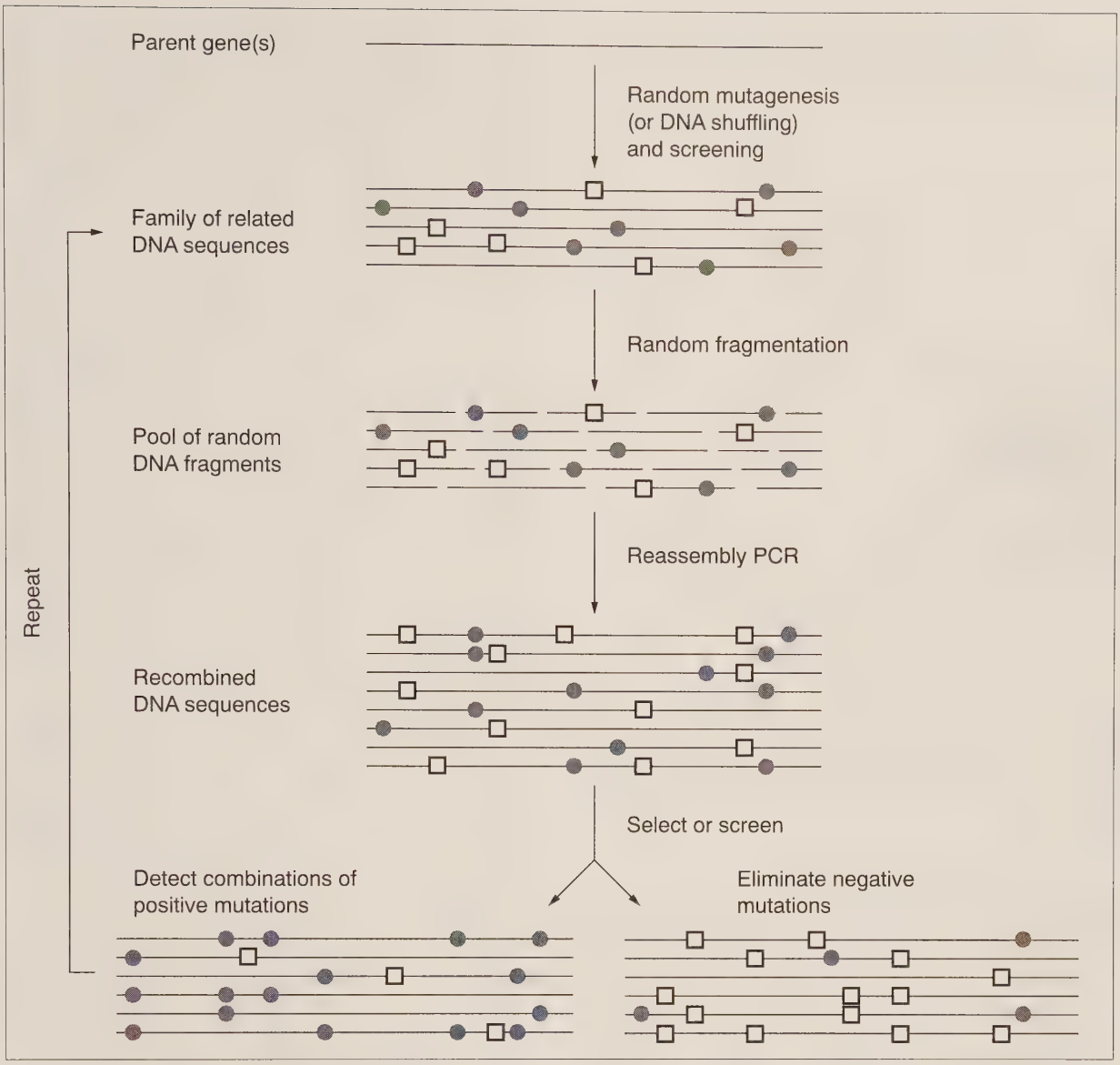


Figure 2

In vitro recombination by DNA shuffling. Random fragments of a single parent gene or pool of genes containing different mutations are reassembled in a PCR reaction, during which they are allowed to act as primers for one another. The fragments elongate, producing a family of DNA sequences containing different combinations of the mutations contained in the parent pool. Beneficial mutations are accumulated and deleterious mutations eliminated during recursive cycles of shuffling and screening or selection (adapted from Ref. 11).

individual mutations resulted in conversions with 85–91% enantiomeric excess.

Techniques and strategies for creating and searching genetic diversity

Efforts to develop and optimize techniques for introducing point mutations randomly into DNA segments facilitated many of the first experiments in directed enzyme evolution. Random mutations have been introduced continuously along a stretch of DNA using error-prone PCR, chemical mutagenesis, UV irradiation, mutator strains, poisoned nucleotides or concomitantly with DNA recombination. Among the methods that introduce mutations randomly along the entire length of a gene, error-prone PCR[36,37] has been characterized the most extensively. The protocol designed by Leung *et al.*[37] provides control of mutagenic rates by varying the concentrations of Mn^{2+} and dNTPs. However, this method exhibits a significant bias for transitions over transversions. The protocol of Joyce and co-workers[36] diminishes this bias but does not eliminate it[2]. Only a fraction of all the possible amino acid substitutions are accessible by point-mutation methods; sequence bias further limits the sequence space.

When a particular set of amino acids has been targeted for mutagenesis based on prior knowledge, region-specific random mutagenesis can be carried out using oligonucleotide-cassette mutagenesis[38,39]. This method is convenient when the targeted amino acids are all in the same region of primary sequence. The groups of Sauer[40], Youvan[41] and Loeb[1] have used this approach extensively to investigate structure–function relationships in enzymes and to probe the information content of amino acid sequences.

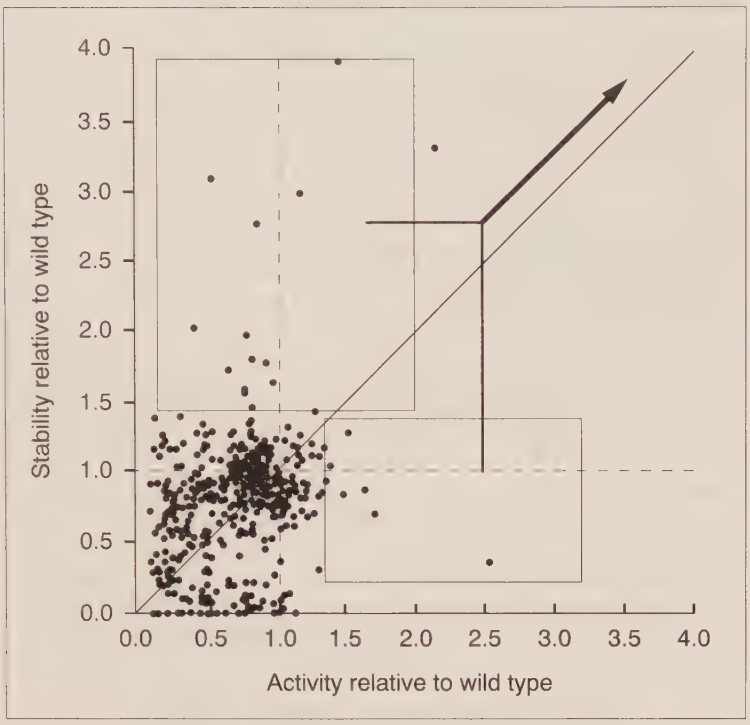


Figure 3

Activity and stability to hydrogen peroxide of a randomly mutagenized enzyme library. Variants exhibiting improvements in single properties are much more frequent than variants with improvements in both properties. The individual populations shown in the boxes can be evolved in parallel and recombined to obtain enzymes that are both more active and more stable.

The DNA-shuffling method developed by Stemmer[42] for random *in vitro* DNA recombination represents a significant advance in our ability to mimic the natural mechanisms of evolution. DNA shuffling (Fig. 2) creates libraries of genes containing combinations of mutations derived from a set of homologous natural sequences or arising as a result of point mutation; minor variations on this method have allowed greater control over the point mutagenesis associated with DNA shuffling[43,44]. A new method for *in vitro* mutagenesis and recombination uses extension of random-sequence oligonucleotides to create the gene fragments for reassembly (Z. Shao, *et al.*, unpublished). The utility of recombination in directed enzyme evolution has been discussed[13,45] and strategies for combining sequential point mutagenesis with recombination explored[13]. Recombination allows the rapid accumulation of beneficial mutations identified in separate genes. Screening protocols, for example, often produce multiple improved sequences. The beneficial mutations normally discarded when a single clone is chosen from these to parent the subsequent generation can be efficiently recombined by DNA shuffling. In addition, delerious mutations are removed.

Recombination is also a promising approach to combining two or more improved properties evolved separately. This concept is illustrated in Fig. 3, which shows the results of screening a randomly mutagenized enzyme library (e.g. of subtilisin) for two separate properties, activity towards a particular substrate and stability towards hydrogen peroxide. Although few variants exhibit improvements in both properties, several are either more stable or more active (sometimes at the cost of the second property). These populations can be recombined in order to create enzymes that are both more stable and more active. With efficient *in vitro* recombination methods, such as DNA shuffling, it should be possible to combine several desired features, starting from libraries of enzymes evolved separately for individual properties.

Delagrave *et al.*[41] applied combinatorial cassette mutagenesis in an approach they call recursive combinatorial mutagenesis. This algorithm uses information about functionally conserved residues learned during each round of combinatorial cassette mutagenesis to guide the next round of oligonucleotide-cassette synthesis. The objective of sampling combinations of several amino acid substitutions at once is similar to the goal of DNA shuffling[42]. In combinatorial cassette mutagenesis, random cassettes are created to replace a limited number of contiguous amino acids, whereas DNA shuffling is appropriate for mutagenizing and recombining much longer sequences.

The decision whether to restrict mutagenesis to a target set of residues can be a difficult one. Limiting the number of amino acids to be mutated greatly reduces the sequence space that must be searched (Box 1), allowing the sampling of multiple simultaneous substitutions in key regions of the enzyme. However, the fact that mutations far from the active site or binding pocket modulate activity, substrate recognition or binding[9,11] underscores the risks inherent in restricting the regions undergoing evolution.

The array of available screening and selection methods has also expanded with the proliferation of directed-evolution experiments. Screening[25] and selection[46] approaches useful for directed enzyme evolution have recently been reviewed. Selections are attractive for searching larger libraries of variants, but are difficult to devise for enzymes that are not critical to the survival of the host organism. Furthermore, organisms are notoriously adept at evading imposed selective pressures by unexpected mechanisms. Less-stringent functional complementation can be very useful for identifying variants that retain biological activity in libraries generated using relatively high mutagenic rates[1,2,25]. In choosing an assay or selection, it is useful to remember the first law of random mutagenesis: you get what you screen for[6].

Local fitness landscapes

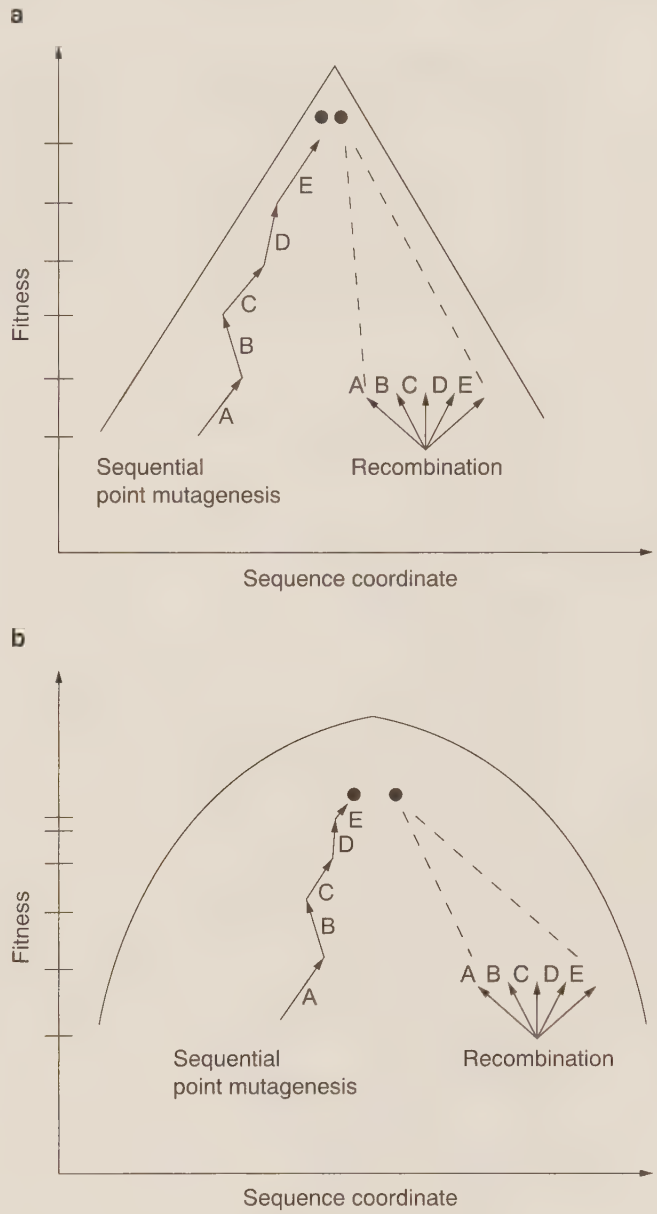
A local fitness landscape is a representation of the fitness – in this context, the extent to which an enzyme exhibits a desired property – of not-too-distant sequence variants of an enzyme. Such landscapes are often greatly simplified for depiction in two or three dimensions (Box 2). (The term 'fitness landscape' often refers to the fitness values of all possible sequences[47,48]. Such a landscape is clearly not accessible by any experimental measures.) Although the

shape of local protein-fitness landscapes is subject to debate[49], the high dimensionality of sequence space, the near-additivity of the free energies of individual mutations for many properties[21] and statistical models[50] all suggest that local landscapes are more 'Fujiyama-like' (i.e. increase more-or-less monotonically towards a fitness optimum) than highly rugged.

Several groups have attempted to bring the concept of protein landscapes to bear on practical issues of devising strategies for directed evolution. Urabe and coworkers[51] created a library of versions of *B. stearothermophilus* catalase I using an average mutagenic rate of one mutation per gene and characterized 82 of these variants for fitness with respect to their activity as a catalase, activity as a peroxidase and thermostability. The profiles generated by plotting each of these characteristics from best to worst were termed 'single-mutant-neighbour' fitness landscapes. Such simplified fitness profiles are very useful for evaluating the feasibility of a particular evolutionary experiment. For example, the position of the fitness of the wild-type or parent enzyme relative to the remainder of the profile provides an indication of the 'evolvability' of a particular trait. The fact that the esterase profile in Fig. 1 shows a number of mutants more stable than the wild-type enzyme suggests that thermostability in this system is highly amenable to directed evolution. Urabe and coworkers reached a similar conclusion for *B. stearothermophilus* catalase I. Parameters such as the proportion of clones that are inactive, easily obtained from fitness profiles, are also very useful for estimating mutagenic rates[2] and optimizing mutagenesis conditions.

Aita and Husimi[50] built a model protein landscape based on the additivity of the free-energy effects of amino acid substitutions and the introduction of functions to describe the tolerance to amino acid substitution at each site. Such tolerance functions have been measured experimentally by Delagrave *et al.*[41] and Suzuki *et al.*[1] Although an *a priori* assumption of additivity automatically prejudices the landscape to be Fujiyama-like, the methods developed by Aita and Husimi[50] allow shape parameters such as the slope of the landscape or the fitness of the global optimum to be estimated from local fitness profiles. Different evolutionary strategies, such as sequential point mutagenesis and recombination, can be depicted on the simplified landscape (Box 2).

Box 2. A depiction of two 'Fujiyama-type' local protein-fitness landscapes with different slope characteristics



Fitness, for our purposes, is the extent to which the enzyme exhibits a desired characteristic. Beneficial mutations (A–E) increase enzyme fitness by an amount equal to the *y* component of the arrow. The landscape can be traversed by sequential point mutagenesis and selection or screening over multiple generations, or by recombination of beneficial mutations identified in earlier generations.

(**a**) A Fujiyama-type landscape with constant slope. The constant slope indicates that the mutations are completely additive in their effects on fitness.

(**b**) A Fujiyama-type landscape with decreasing slope approaching the fitness optimum. Here, the benefit of any given mutation depends on the rest of the sequence. Mutations in advanced generations are more difficult to identify owing to diminishing returns obtained for each mutation. Recombination of point mutations from earlier generations also yields a smaller increment in fitness than strict additivity would predict.

The complete landscape is multidimensional and includes fitnesses for all possible sequences and pathways. The 'sequence coordinate' is a one-dimensional representation of all possible amino acid combinations.

Future directions

The first *in vitro* evolution of a metabolic pathway was recently reported by Crameri *et al.*[12], who evolved a three-gene operon coding for arsenic resistance. Several rounds of DNA shuffling were used to achieve a 40-fold increase in arsenate detoxification in the absence of any structural information or knowledge of which gene products are rate-limiting. Extending the power of directed evolution to groups of genes offers a glimpse of exciting future avenues for enzyme and metabolic engineering. A trend toward further miniaturization of assay formats and higher-throughput automation of screening promises to increase the libary

sizes accessible to searching. Increasing control over mutagenic methods and insight into optimal strategies for traversing protein landscapes will further expand the potential of directed evolution to create novel biocatalysts.

Acknowledgments

Support from the Army Research Office, the Office of Naval Research and the Department of Energy's program in Biological and Chemical Technologies Research is acknowledged. O. Kuchner is the recipient of an NIH predoctoral training fellowship from the National Institute of General Medical Sciences, NRSA Award 1 T32 GM 08346-01. The authors thank Daniel Song for helpful discussions.

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Reasons to use maths

Nonlinear Time Series Analysis

by H. Kantz and T. Schreiber, Cambridge University Press, 1997. UK£50.00 hbk (xvi + 304 pages) ISBN 0 521 55144 7

What, you may ask, has time-series analysis to do with biotechnology? This book provides a clear answer, albeit implicitly, in that over half of the applications described use biological data – from atrial fibrillation, electrocardiograms and breathing rates to phonation and posture control. The new techniques of nonlinear time-series analysis are found to give considerable insight. For example, they allow one to extract the tiny foetal electrocardiogram component from electric potentials on the abdomen of a pregnant woman, a feat that the authors claim cannot be achieved by classical linear techniques.

The basic principle is to suppose that the observations have a deterministic origin and to extract as much information as possible about the assumed underlying deterministic dynamical system. Although one cannot expect to extract a unique model for the underlying process, any feature of the dynamical system that is invariant under a smooth change of coordinates on the state space can be computed. This includes a wide range of quantities, such as 'Lyapunov exponents', 'fractal dimensions' and 'entropies'. Furthermore, the approach can be used to subtract out measurement noise, to make predictions about future observations and to devise control schemes that turn chaotic into periodic behaviour.

The authors give a very readable introduction to the concepts, and clear descriptions of the techniques, illustrated by applications to laser physics and biotechnology – this book is probably the best place I know of to learn this material. It requires some facility with mathematics, but should be accessible to anyone with an undergraduate 'Mathematics for Scientists' background. The authors are also careful to give cautions, where appropriate, about potential pitfalls in interpreting the results and misuses of the methods. For instance, they give a particularly nice discussion of the occurrence of spurious Lyapunov exponents.

I have three main criticisms. Firstly, what do we do with Lyapunov exponents, dimensions and entropies once we have extracted them from a time series? I do not know a good answer to this, and it seems to me that the main benefits of the nonlinear-time-series-analysis approach lie elsewhere. For example, in collaboration with SRAMA (a consortium of spring manufacturers in Sheffield), I. Stewart and M. Muldoon at the University of Warwick designed a successful test for wire quality, based on nonlinear time-series analysis ('time' being length along the wire in this application), using none of these dynamical systems invariants but rather the 'shape' of the recurrent set.

Secondly, there are other coordinate-free features of dynamical systems that can be extracted but are not mentioned. For example, one can extract the number of independent frequencies of a quasiperiodic signal, or, more generally, the Betti numbers of the attractor. Similarly, one can compute the Conley index of the attractor from measurement of transients, and for three-variable systems one can extract knot types and linking invariants. These topological quantities could be equally useful.

Thirdly, many of the procedures are ad hoc: they seem to me to be recipes with some motivation but little guiding philosophy. I would suggest that the whole subject of nonlinear time-series analysis would benefit greatly from the theory of Bayesian inference, which gives a consistent framework for updating the evidence for models of stochastic processes that generate a time-series, in the light of new data. This allows the evidence for different models to be compared, to obtain, in particular, a posterior probability distribution over parameter spaces. An advantage of the method is that it intrinsically penalizes over-complicated models. The results can be used to make predictions by integrating extrapolations of the models over the posterior distribution, and the method has been used very successfully to make cross-predictions when some variables remain observable and others do not. For models of the special form 'deterministic dynamical system plus measurement noise', as is implicitly assumed in most of this book, the method can produce a probability distribution over the deterministic trajectories, and hence an excellently controlled noise-reduction procedure and a handle on whatever features of the deterministic dynamical system are desired. It would be nice to see these ideas developed.

Despite these three criticisms, I can recommend the book to anyone involved in the interpretation of time series, as a good presentation of an important development in the area.

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PII: S0167-7799(97)01146-3

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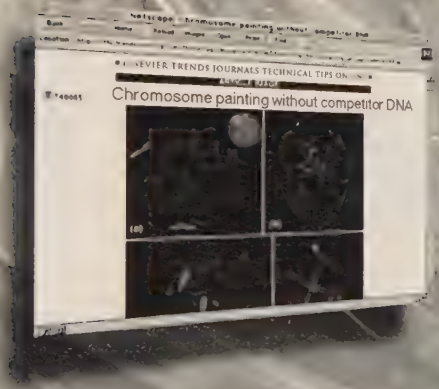

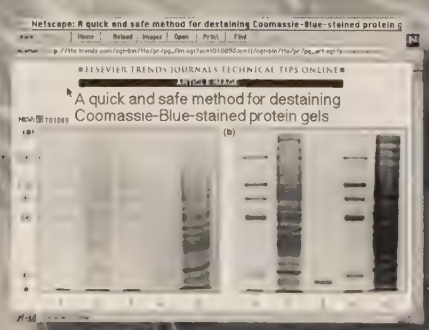

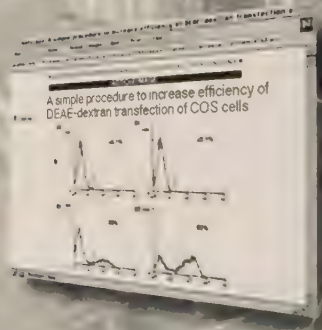

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TP 248.13 .T74 v.15 1997
Trends in biotechnology